THE ROLE OF SERUM IgM IN IMMUNITY AND AUTOIMMUNITY

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SUMMARY

Natural IgM has a wide range of actions in the immune system, is the first class of antibody to be produced, provides immediate defence against infection and assists subsequent development of the immune response. IgM is a potent complement activator, because of its pentameric structure and can have stable interactions with polymeric antigens. Here I investigated the effects of serum IgM deficiency on B cell development. Mice lacking serum IgM (Sμ-) have an expansion in marginal zone (MZ) B cells with a corresponding reduction in follicular (FO) B cells. This expansion in MZ B cells is further accentuated in response to T-independent antigens. I have found that the increase in MZ/FO B cell ratio (and the expansion of peritoneal B1 cells) is fully reversed by administration of polyclonal, but not monoclonal, IgM. Natural IgM, by virtue of its polyreactivity, may enhance antigen driven signalling through the B cell receptor (BCR) and promote the formation of FO B cells. Conversely, in the absence of serum IgM, BCR signalling is reduced and MZ B cells are preferentially formed. In the absence of serum IgM, splenic follicular B cells have a shortened life span whereas peritoneal B1 B cells have a longer life span. However proliferation, and calcium mobilisation in response to BCR crosslinking in vitro is normal. These results demonstrate that natural IgM regulates the selection of B lymphocyte subsets in the periphery.

It was previously shown that Sμ- mice have an increased propensity for developing IgG autoantibodies. In order to determine whether serum IgM deficiency accelerates autoimmunity, Sμ- mice were intercrossed into C57Black/6*lpr* mutant mice, which develop mild autoimmunity and lymphadenopathy. It was found that splenic marginal zone B cell numbers were further increased in the *lpr* serum IgM deficient (Sμ-*lpr*) mice. This increase in MZ B cells is associated with more severe lymphadenopathy, but not with worsening autoimmunity. Furthermore, the autoimmunity observed in these mice does not appear to be as a direct consequence of MZ B cell expansion.

DECLARATION

I certify that this thesis does not incorporated any material previously submitted for a degree or diploma in any university, and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Monica for looking after Noah.

TO NOAH FOR BRINGING LIGHT

AND LAUGHTER TO MY LIFE

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

APC- antigen presenting cell

Baff- B cell activating factor of the TNF family

BCR- B cell antigen receptor

BrdU- 5-bromo-2'-deoxyuridine

BSA- Bovine serum albumin

C- Constant

CD- Cluster of differentiation

D- Diversity

dsDNA- double stranded DNA

ELISA- Enzyme linked immunosorbent assay

FACS- Fluorescence activated cell sorter

FCS- Fetal calf serum

FDC- Follicular dendritic cells

FITC- Fluorescence isothyiocyanate

FO- Follicular

H- Heavy

HEL- Hen egg lysozyme

HRP- Horse radish peroxidase

HSA- Heat stable antigen

Ig- Immunoglobulin

IL7R- Interleukin 7 receptor

ITAM- Immunoregulatory tyrosine activation motifs

J- Joining

L- Light

LPS- Lipopolysaccharide

MHC- Major histocompatibility complex

MZ- Marginal zone

NP- 4-hydroxy-3-nitrophenylacetyl

PALS- Periarteriolar lymphocyte sheath

PBS- Phosphate buffered saline

PC- Phosphorylcholine

PCR- Polymerase chain reaction

PE- Phycoerytherin

T1- Transitional type 1

T2- Transitional type 2

TG- Transgenic

TI- Thymus independent

TLR- Toll like receptor

TUNEL- Terminal dUTP nucleotide end labeling

V- Variable

INTRODUCTION

INTRODUCTION

Immunoglobulins

Each B cell is genetically programmed to encode a surface receptor specific for a particular antigen. The surface immunoglobulin (Ig) is the B cell antigen receptor (BCR), which has two main roles, firstly to transmit signals that regulate B cell fate decisions and secondly to mediate antigen processing, leading to presentation of antigen to T cells, which allows full activation of B cells in the effector phase.

The BCR is composed of the ligand recognising Ig heavy and light chains in a noncovalent association with Igα and Igβ transmembrane proteins. Igα and Igβ are associated as disulfide-linked heterodimers and mediate the signal transduction function of the BCR. Antigen mediated aggregation of BCR complexes is required to trigger and sustain the signaling processes necessary for generating a signal of sufficient strength and duration to elicit responses by immature or mature B cells. Aggregation triggers an intracellular signaling cascade that requires immunoregulatory tyrosine activation motifs (ITAMs) present on the cytoplasmatic domains of Igα and Igβ (Wienands, 2000).

Immunoglobulins are remarkable not only for the diversity of their antigen-binding sites, but also for their versatility as effector molecules. Immunoglobulins consist of two heavy (H) chains (each with four domains)

and two light (L) chains (κ or λ, each with two domains). The specificity of an antibody response is determined by the antigen-binding site, which consists of the two variable (V) domains, VH and VL. The effector action of the antibody is determined by the isotype of its heavy-chain constant (C) region. The heavy-chain variable (V) domain can become associated with the C region of any isotype through the process of isotype switching, which involves VD (diversity) J (joining) recombination. The five isotypes of immunoglobulin are IgM, IgD, IgG, IgE, and IgA. In the mouse, IgG antibodies can be further subdivided into four subclasses (IgG1, IgG2a, IgG2b and IgG3). The functions and properties of the different subclasses of human immunoglobulins are summarised on Table 1, and Figure 1 shows the transmembrane and the secreted from of immunoglobulins (Janeway et al, 2001).

Table 1. The properties of the human immunoglobulin isotypes. IgM is so called because of its size: although monomeric IgM is only 190 kDa, it normally forms pentamers, known as macroglobulin (hence the M), of very large molecular weight. IgA dimerises to give a molecular weight of around 390 kDa in secretions. IgE antibody is associated with immediate-type hypersensitivity. When fixed to tissue mast cells, IgE has a much longer half-life than its plasma half-life shown here (Janeway et al, 2001).

	Immunoglobulin								
	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Heavy chain	γ_1	γ_2	γ_3	γ_4	μ	α_1	α_2	δ	ε
Molecular weight (kDa)	146	146	165	146	970	160	160	184	188
Serum level (mean adult mg ml^{-1})	9	3	1	0.5	1.5	3.0	0.5	0.03	5×10^{-5}
Half-life in serum (days)	21	20	7	21	10	6	6	3	2
Classical pathway of complement activation	++	+	+++	−	+++	−	−	−	−
Alternative pathway of complement activation	−	−	−	−	−	+	−	−	−
Placental transfer	+++	+	++	−/+	−	−	−	−	−
Binding to macrophages and other phagocytes	+	−	+	−/+	−	+	+	−	+
High-affinity binding to mast cells and basophils	−	−	−	−	−	−	−	−	+++
Reactivity with staphylococcal Protein A	+	+	−/+	+	−	−	−	−	−

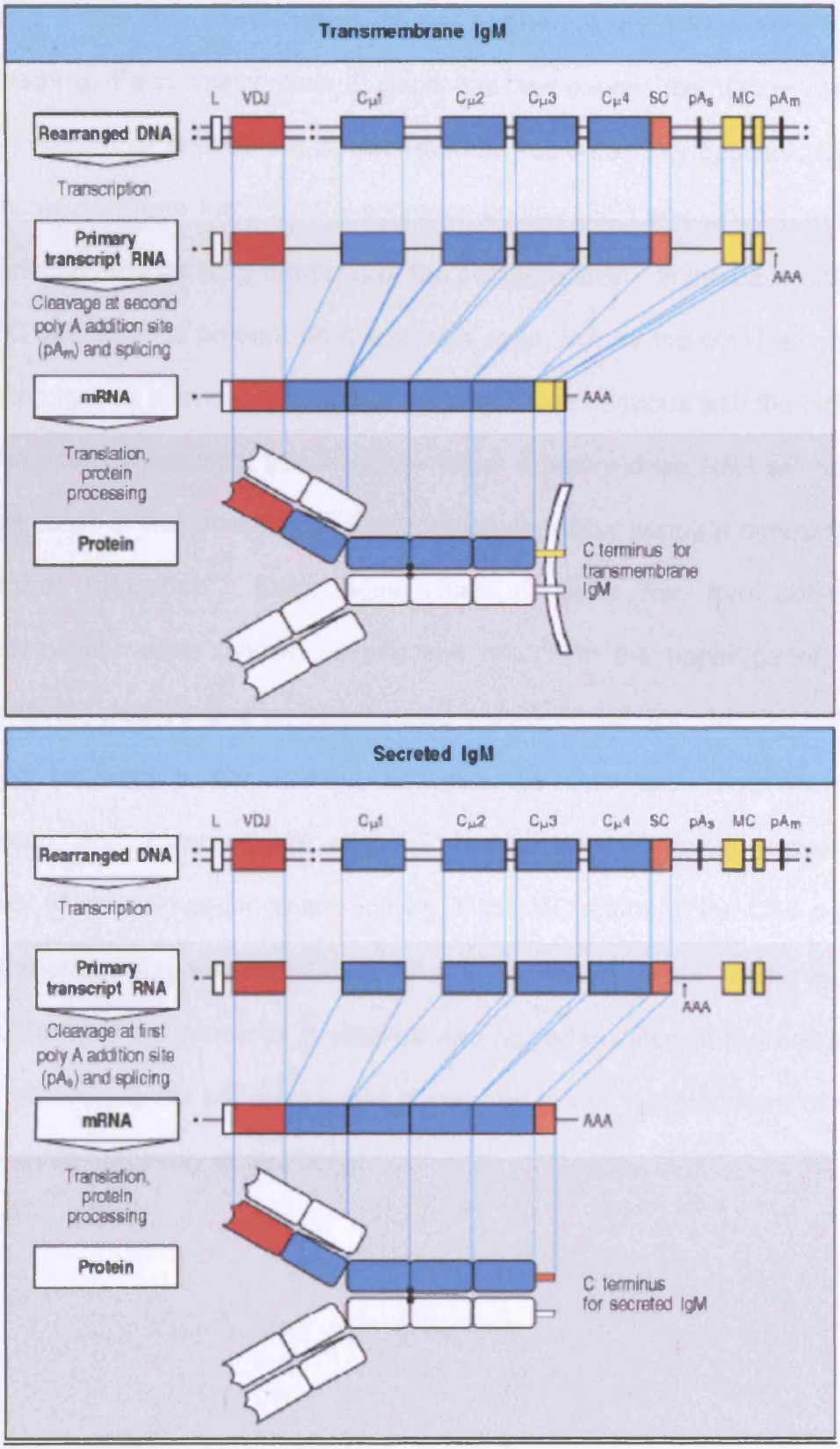
Transmembrane IgM
L
VDJ
Cμ1
Cμ2
Cμ3
Cμ4
SC
pAs
MC
pAm
Rearranged DNA
Transcription
Primary transcript RNA
AAA
Cleavage at second poly A addition site (pAm) and splicing
mRNA
AAA
Translation, protein processing
Protein
C terminus for transmembrane IgM
Secreted IgM
L
VDJ
Cμ1
Cμ2
Cμ3
Cμ4
SC
pAs
MC
pAm
Rearranged DNA
Transcription
Primary transcript RNA
AAA
Cleavage at first poly A addition site (pAs) and splicing
mRNA
AAA
Translation, protein processing
Protein
C terminus for secreted IgM

Figure 1. Transmembrane and secreted forms of immunoglobulins are derived from the same heavy-chain sequence by alternative RNA processing. Each heavy-chain C gene has two exons (membrane-coding (MC) yellow) that encode the transmembrane region and cytoplasmic tail of the transmembrane form, and a secretion-coding (SC) sequence (orange) that encodes the carboxy terminus of the secreted form. In the case of IgD, the SC sequence is present on a separate exon, but for the other isotypes, including IgM as shown here, the SC sequence is contiguous with the last C-domain exon. The events that dictate whether a heavy-chain RNA will result in a secreted or transmembrane immunoglobulin occur during processing of the initial transcript. Each heavy-chain C gene has two potential polyadenylation sites (shown as pA_s and pA_m). In the upper panel, the transcript is cleaved and polyadenylated (AAA) at the second site (pA_m). Splicing between a site located between the Cμ4 exon and the SC sequence, and a second site at the 5' end of the MC exons, results in removal of the SC sequence and joining of the MC exons to the Cμ4 exon. This generates the transmembrane form of the heavy chain. In the lower panel, the primary transcript is cleaved and polyadenylated at the first site (pA_s), eliminating the MC exons and giving rise to the secreted form of the heavy chain (Janeway et al, 2001).

The IgM molecule is a pentamer of 4-chain structure. Each heavy chain has a molecular weight of approximately 65000 and the whole molecule has a molecular weight of 970000. The μ chains of IgM have an extra constant region domain. The sub-units of the pentamer are linked by disulphide bonds between the Cμ3 domains, and possibly by disulphide bonds between the C terminal 18 residue peptide tailpiece. The complete molecule consists of a densely packed central region with radiating arms (Figure 2). The dislocation resulting in the "crab like" configuration appears to be related to the activation of complement by IgM (Turner et al, 1998).

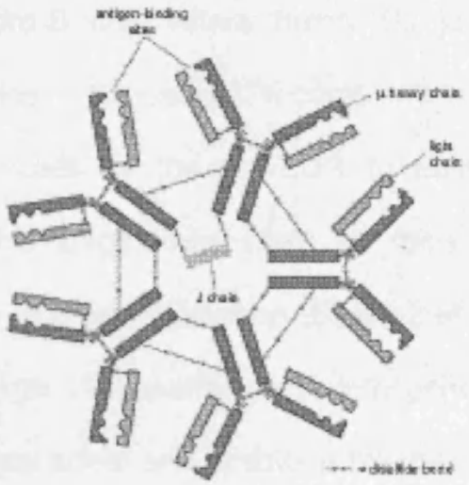

Figure 2. A pentameric IgM molecule. The five subunits are held together by disulfide bonds. A single J chain, which has a structure similar to that of a single Ig domain, is disulfide-bonded between two μ heavy chains. The J chain is required for the polymerization process. The addition of each successive four-chain IgM subunit requires a J chain, which is then discarded, except for the last one, which is retained. (Alberts et al, 1994).

B cell development

In the mouse B cells are generated from pluripotent hematopoietic stem cells in the liver during mid to late fetal development and in the bone marrow after birth. In addition to being a site for B cell development, the postnatal bone marrow contains mature T cells and plasma cells.

After commitment to B cell lineage in the bone marrow, developing murine B cells require signals through both their B cell antigen receptor (BCR) and interleukin 7 receptor (IL7R) to allow differentiation and survival. BCR generation by VDJ recombination of immunoglobulin heavy and light chains initiates in pro-B cell, where heavy DJ joins are initially made, followed by VDJ joins. Successful recombination allows feedback and expansion of pro-B cells by the pre-BCR to enter the pre-B stage of development. At this stage, light chain VJ joins are made and allelic exclusion terminates VDJ recombination (Bassing et al, 2002). B cells that fail to properly undergo VDJ rearrangement to generate a functional BCR undergo developmental arrest and apoptosis (Nemazee et al, 2000).

During B cell development, immature B cells developing in the bone marrow are the first cells to express the prototypic form of the BCR, consequently the immature B cell is the first representative of the B cell to recognise and respond to antigen in a clonotypically restricted manner, thus the immature B cell is of critical importance to the immune system, for it is at this stage that antigen specific positive and negative selection events initially

operate. Such selective events exert a profound influence on the generation of the peripheral mature B cell repertoire (King et al, 2000).

An adult mouse produce about $2x10^7$ of immature B cells per day (Osmond, 1991) and only around 10%-20% of the immature B cells made in the bone marrow migrate to the spleen (Allman et al, 2001, Rolink et al, 1998). The loss of the initial immature B cells may be due to several types of negative selection (receptor editing, clonal deletion and anergy) to avoid autoreactivity. Most of the work done on negative selection of B cells in the bone marrow has been done on transgenic models, the one mostly used is the HEL (hen egg lysozyme) system, where B cells express transgenic immunoglobulin receptors for HEL (MD4 anti-HEL Ig [IgHEL]) (Hartley et al, 1991, Goodnow et al, 1989, Goodnow et al, 1988 and Cooke et al, 1994).

Receptor editing operates at genetic level, replacing expressed defective and/or self-reactive variable (V) genes with upstream V genes coding for hopefully harmless receptors (Gay et al, 1993; Radic et al, 1993 and Tiegs et al, 1993). The re-arrangement of heavy (H) and/or light (L) chain genes are induced by encounters with auto-antigens to change specificity from self to non self.

Another form of negative selection is by clonal deletion by apoptosis of self-reactive immature B cells that are capable of crosslinking immunoglubulin receptors on B cells (BCR) with high avidity in the bone marrow (Hartley et al, 1991, Nemazee et al, 1989, Okamoto et al, 1992 and Sandel et al, 1999). Self-reactive B cells expressing transgenic Ig receptors

for HEL undergo deletion or receptor editing in the bone marrow when they encounter systemic membrane-bound HEL (mHEL) expressed on the cell surface under the class I promoter (Hartley et al, 1991).

Immature self-reactive B cells can also become functionally inactivated (anergic), but are not physically eliminated. IgHEL B cells that encounter abundant but lower avidity soluble HEL (sHEL) at a level in excess of 10–20 ng/ml enter the repertoire but are held in a potentially reversible state of functional inactivation (Goodnow et al, 1989, Goodnow et al, 1988 and Cooke et al, 1994) and have a shortened life span which is largely due to their inability to compete with other B cells (Cyster et al, 1994 and Cyster et al, 1995). Autoreactive B cells that escape tolerisation in the bone marrow are eliminated on peripheral encounter with autoantigen (Loder et al, 1999 and Russell et al, 1991), whereas B cells that bind low avidity or rare antigens may remain functionally ignorant (Aldelstein et al, 1991 and Hannum et al, 1996). When B cells emerge from bone marrow into the periphery, they are still functionally immature, expressing high levels of surface IgM but little surface IgD (Janeway et al, 2001). Figure 3 briefly illustrates negative selection in the bone marrow.

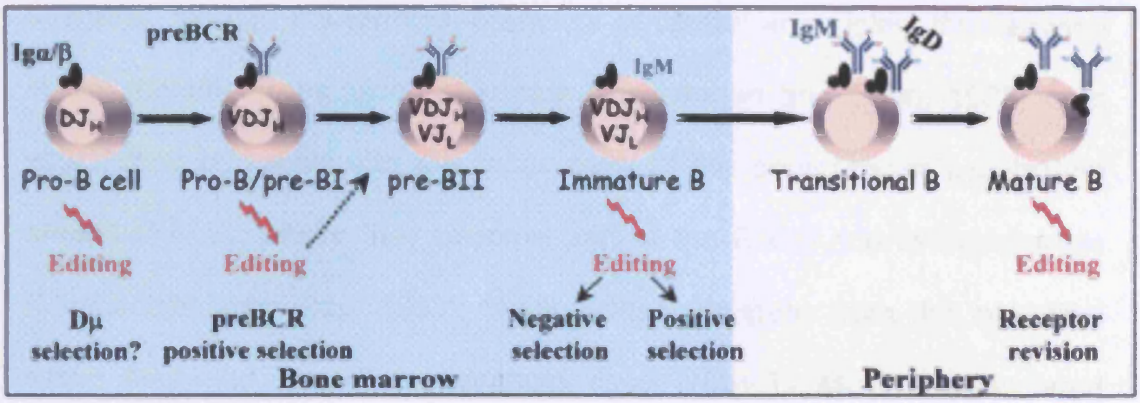

Figure 3. B cell development. Receptor editing is activated throughout B lymphopoiesis. Selection checkpoints mediated by the pre-BCR or the BCR are specified. B cells that fail to fulfil appropriate receptor requirements stimulate receptor editing and undergo secondary recombination (Edry et al, 2004).

B cell development also depends on BCR expression and appropriate signaling, a process referred to as positive selection. Positive selection in the bone marrow takes place at the immature stage during the process of entry into the more stable peripheral B cell pool. This selection happens by antigens/autoantigens binding to the heavy chain V region independently of the light chain (Gu et al, 1991) to enrich the repertoire for functional diversity. Mice in which the BCR signaling is impaired by the deletion of syk tyrosine kinase (Turner et al, 1997) or by the truncation of Igα (Torres et al, 1996) show a marked reduction in the transition from immature B cells from the bone marrow to the spleen.

The generation of lymphocytes in primary lymphoid organs is followed by their migration into peripheral secondary tissues; spleen (blood borne antigens) through the terminal branches of central arteorioles, thus arriving in the marginal zone blood sinusoids (MacLennan and Chan, 1993) from which they penetrate into the outer zone of the periarteriolar lymphocyte sheath (PALS), where they become part of the B cell rich follicular areas (MacLennan and Gray, 1986); lymph nodes (antigens from the interstitial tissue fluid and lymph) or lymphoid tissue (MALT- Mucosa Associated Lymphoid Tissue) (Lydyard and Grossi, 1998).

The spleen is divided into two parts, the white pulp and the red pulp. The white pulp consists of lymphoid tissue, arranged around a central arteriole to form the periarteriolar lymphoid sheaths (PALS). PALS are composed of T and B cell areas. B cells are organised into either primary (unstimulated) follicles, which aggregate naive B cells, or secondary (stimulated) follicles. Secondary follicles possess a germinal centre, an area in which B cells involved in the immune response are proliferating and undergoing somatic hypermutation and also contain memory B cells. T cell zones and follicles are surrounded by marginal zones, containing a unique population of B cells, the marginal zone B cells (MZ), which do not re-circulate. The marginal zone is adjacent to the red pulp and separated from the follicles by a loose endothelial structure, the marginal sinus, as seen in figure 4 (Lortan et al, 1987).

The marginal zone also contains dendritic cells as well as two different types of macrophages, one of which lines the marginal sinus (the so-called 'metallophilic macrophages') whereas the other is scattered throughout the marginal zone. The B cell zone contains a network of follicular dendritic cells (FDCs), which are mainly concentrated in the area of the follicle most distant from the central arteriole (Figure 4). Follicular dendritic cells differ from the dendritic cells, as they are not leukocytes and are not derived from bone marrow precursors. They are not phagocytic and do not express MHC class II proteins. Follicular dendritic cells seem to be specialised to capture antigen in the form of immune complexes. The immune complexes are not internalised; they remain intact on the follicular dendritic cell surface, where the antigen can be recognised by B cells. Follicular dendritic cells are also important in the development of B cell follicles (Janeway et al, 2001).

Lymph nodes consist of a B cell area (cortex) and T cell area (paracortex), and a central medulla, formed by cellular cords containing macrophages, T, B and lymphocytes and plasma cells (Lydyard and Grossi, 1998).

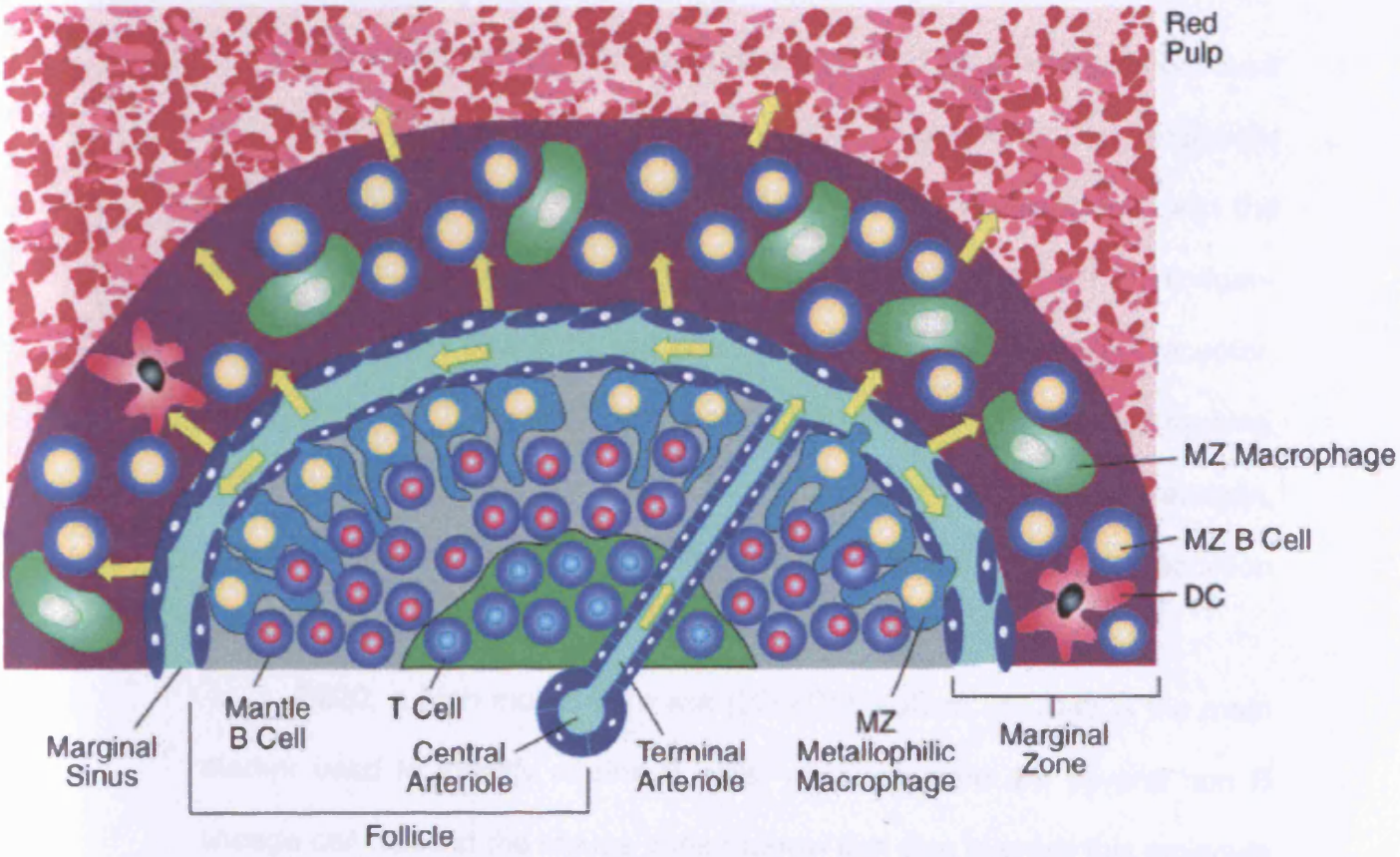

Figure 4. Structure of the marginal zone. Blood from the central circulation moves from the central to terminal arterioles before emptying into the marginal sinus from which it percolates into the red pulp (arrows). Particulate and soluble antigens acquired by macrophages and dendritic cells are presented to small number of MZ T cells (not shown) and MZ B cells eliciting rapid responses (Morse et al, 2001).

B cell markers

The molecule CD19 characterises most of B lineage stages, because is expressed from early pre-B cells at the time of heavy chain rearrangement until plasma cell differentiation (Li et al, 1996). CD19 associated with the complement receptor for C3d (CD21) plays an important part in antigen-induced B cell activation via the antigen binding antibody receptor. Antibodies already bound to antigens can activate the complement system. When the antigen is coated with C3d, it produces a more potent antigen, which in turn leads to more efficient B-cell activation and antibody production (Janeway et al, 2001).

B220, a high molecular mass (220kDa) isoform of CD45 is the main marker used to identify murine B cells, although there are several non B lineage cell types in the mouse bone marrow that also express this molecule (Rolink et al, 1996).

Immature and mature B cells can be identified by the intensity of cell surface heat-stable antigen (HSA), also known as CD24, immature B cells expressing high levels and mature B cells low levels of HSA (Allman et al, 1992 and Allman et al, 1993).

Follicular and T2 B cell express high levels of the low affinity Fcε receptor (FcεRII) CD23, a C-type lectin (Janeway et al, 2001).

Marginal Zone B cells express high levels of the MHC class I-like molecule CD1d, which has a specialized role in presentation of lipid antigens.

Complement receptors for C3b (CR1, CD35) and C3d (CR2, CD21) are commonly found on T2, FO and MZ B cells and are associated with activation and possibly, homing of the cells (Li et al, 1996).

B1 cells (foetal B cell development) expresses CD5+ and B2 or conventional B cells (generated in adult bone marrow) do not express CD5. B1 phenotype B cells are further subdivided into B1a (CD5 +ve) and B1b (CD5 -ve) (Hardy et al, 2001).

Table 2 shows most of the molecules that are expressed on the surface of B cells.

Table 2. Murine CD antigens

CD antigens	*Cellular expression*	*Functions*	*Other names*	*Family relationships*
CD1d	T cells, B cells, dendritic and NK cells	Antigen presentation. Glycoprotein with structural homology to MHC class I	CD1.1, Ly-38	Immunoglobulin
CD5	T and a subset of B cells (B1a B cells).	Ligand of CD72	T1, Ly1	Scavenger receptor
CD19	B cells	Forms complex with CD21 (CR2) and CD81 (TAPA-1); co-receptor for B cells. Cytoplasmic domain binds cytoplasmic tyrosine kinases and PI 3-kinase		Immunoglobulin
CD21/CD35	Mature B cells, follicular dendritic cells and macrophages	Receptor for complement component C3d, Epstein-Barr virus. With CD19 and CD81, CD21 forms co-receptor for B cells	CR2 and CR1	Complement control protein (CCP)
CD22	Mature B cells and plasma cells	Negative regulation of B cell activation	Lyb-8.2	Immunoglobulin
CD23	Mature B cells, activated macrophages, eosinophils and follicular dendritic cells	Low-affinity receptor for IgE, regulates IgE synthesis; ligand for CD19:CD21: CD81 co-receptor	IgE Fc receptor	C-type lectin
CD24	B cells and granulocytes	Ligand of CD62P (P-selectin)	Heat stable antigen	Membrane protein
CD40	B, monocytes /macrophage and follicular dendritic cells	Binds to CD154 (CD40L), co-stimulatory molecule		TNF receptor
CD45/B220	B cells, abnormal T cells and NK cells	Tyrosine phosphatase, augments signaling through antigen receptor of B and T cells	Leukocyte common antigen (LCA), Ly-5 or T200 and B220	Transmembrane Protein Tyrosine Phosphatase

Pharmingen Catalog 2002 and Janeway et al, 2001)

B cell sub-populations

B cells can be subdivided in the bone marrow using different cell markers and looking at their immunoglobulin gene rearrangement status (Osmond et al, 1990).

Table 3. B cell developmental stages in the bone marrow

Developmental stage	Hardy's nomenclature	Cell markers
Early pro B	Hardy's Fraction A	AA4.1 $B220^{low}$ CD43 $CD24^{low}$
Intermediate pro B	Hardy's Fraction B	CD19 AA4.1 $B220^{low}$ CD43 $CD24^{low}$
Late pro B	Hardy's Fraction C	AA4.1 $B220^{low}$ CD43 $CD24^{low}$ CD19
Large pre B	Hardy's Fraction C'	AA4.1 $B220^{low}$ CD43 $CD24^{high}$ CD19 IgM^{high}
Small pre B	Hardy's Fraction D	AA4.1 B220 $CD24^{high}$ CD19 IgM^{high}
Immature	Hardy's Fraction E	AA4.1 B220 CD43 $CD24^{high}$ CD19 IgM^{high} IgD^{low}

(Hardy et al, 2001 and Cancro et al, 2004).

B cells in the periphery have also been divided into sub-populations depending on their cell surface markers and phenotype. After migrating from the bone marrow to the spleen, immature B cells pass through two

transitional stages. They are known as transitional B cells type I (T1 or newly formed cells-NF), transitional B cells type 2 (T2), before differentiating into naïve mature B cells.

Marginal Zone (MZ) B cells can be generated from T2 or maybe from mature B cells.

Mature or follicular (FO) B cells can be generated from T1 or T2 B cells (Figure 5).

B cells can be distinguished using molecular makers;

T1: B220 ^{low} CD19 IgM^{high} IgD^{low} CD21^{low} CD23^{neg} CD24^{high}

T2: B220 ^{low} CD19 IgM^{high} IgD^{high} CD21^{high} CD23^{pos} CD24^{high}

FO: B220^{high} CD19 IgM^{low} IgD^{high} CD21^{intermediate} CD23^{high} CD24^{low}

MZ: B220 CD19 IgM^{high} IgD^{low} CD21^{high} CD23^{neg} CD24^{high}

B1: B220 CD19 IgM^{high} IgD^{low} CD23^{neg} CD24 CD5

(Allman et al, 2001, Loder et al, 1999, Samardzic et al, 2002, Li et al, 2001 and Su et al, 2002a)

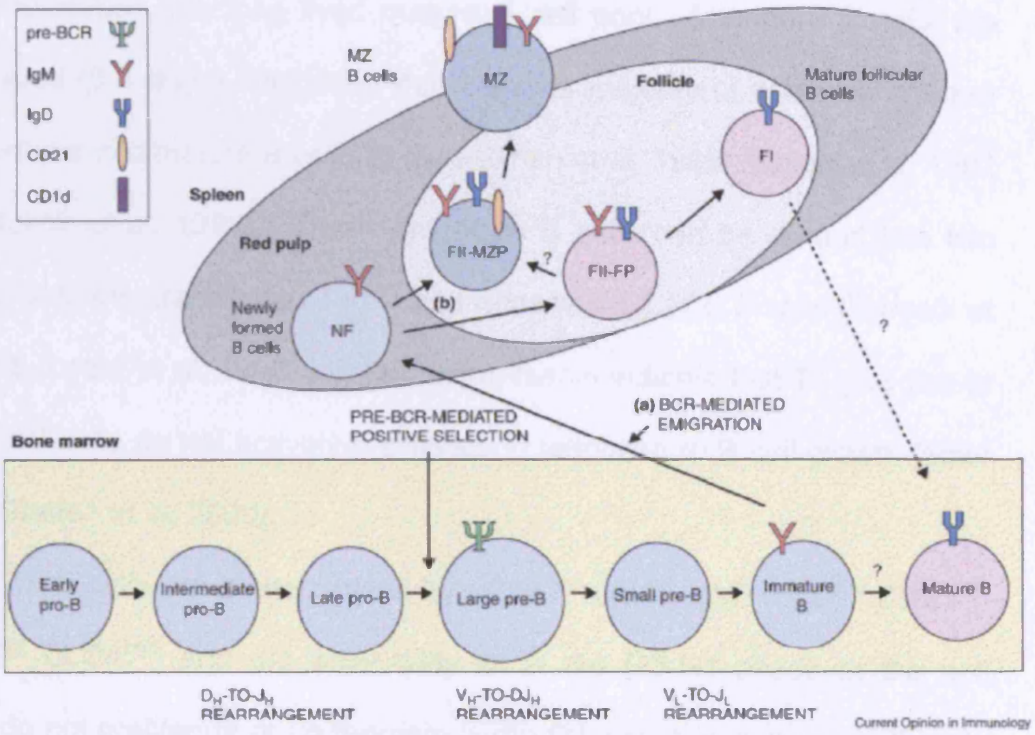

Figure 5. An overview of peripheral B-cell development. B-cell progenitors sequentially rearrange their Ig heavy- and light-chain genes in the bone marrow and, subsequently, **(a)** immature B cells emigrate to the spleen where they can be initially identified as newly formed (NF) B cells. **(b)** Newly formed B cells enter the follicle where they may be initially found either as $IgM^{hi}IgD^{hi}CD21^{int}$ precursors (FII-FP) of mature follicular B cells or $IgM^{hi}IgD^{hi}CD21^{hi}$ precursors (FII-MZP) of MZ B cells. It is unclear whether FII-MZP cells are derived from FII-FP cells. MZ B cells ($IgM^{hi}IgD^{-}CD21^{hi}CD1d^{hi}$; MZ) are long-lived B cells whereas mature follicular B cells ($IgD^{hi}IgM^{lo}$; FI) have lifespan of a few months. B1 B cells are not included in this scheme (Cariappa and Pillai, 2002).

Transitional type 1 B cells

BCR signaling in immature B lymphocytes in the spleen is required for their maturation into long lived mature B cell pool. Immature B cells are short-lived (3-4 days), indicating that the vast majority of these cells either differentiate into mature B cells or die (Allman et al, 1993, Allman et al, 1992 and Rolink et al, 1998). These immature B cells can be divided into two distinct subsets: transitional 1 (T1) and transitional 2 (T2) B cells (Carsetti et al, 1995, Loder et al, 1999). In vivo experiments indicate that T1 give rise to T2. T1 B cells do not actively proliferate in response to B cell growth factor (Baff) (Batten et al, 2000).

T1 B cells are characterised by B220 ^{low} CD19 IgM^{high} IgD^{low} CD21^{low} CD23^{neg} CD24^{high} and are essentially all in the G0-G1 phase of the cell cycle, do not proliferate or up regulate cyclin D2, which is a critical cell cycle regulator in response to antigen receptor activation. BCR stimulation does not rescue T1 cells from death, and instead enhance apoptosis. This may be due to the lack of induction of anti-apoptotic factors such as A1/BFL-1 and the proto-oncogene Akt/PKB, which is important for a range of cellular growth and survival signals. The T1 subpopulation is thought to be a target for BCR induced negative selection in the periphery and act as a distinct checkpoint for selection into the mature B cell pool whereby T1 B cells with receptor specificities for blood-borne self antigens are deleted by negative selection (Su et al, 2002a).

T1 B cells are located in the bone marrow, blood and spleen at the outer periarteriolar lymphoid sheat (PALS) outside of the follicle (Loder et al, 1999). T1 cells migrating to the spleen expressing antigens receptors with a high affinity to soluble antigens in the blood are likely to die via antigen induced apoptosis. Upon entry into the spleen, T1 cells remain in the PALS, where additional blood borne self-antigens trapped by the spleen may further drive negative selection. The remaining T1 cells enter the primary follicle and become T2 cells (Su et al, 2004).

Mice lacking the cytoplasm domain of Igα (Mb1;CD79a) do not have T1 cells, suggesting that T1 cells require a low threshold tonic BCR signal to generate the T2 subset (Torres et al, 1996 and Loder et al, 1999).

Transitional type 2 B cells

Transitional type 2 B cells are situated within primary follicles adjacent to mature B cells (Loder et al, 1999), and are likely to encounter a unique set of antigens, possibly on follicular dendritic cells (Su et al, 2004). They can process and present antigens as peptide:MHC class II complexes, however their ability to activate T cells and elicit help signals is compromised compared to follicular B cells (Chung et al, 2003). Most T2 are in G2/M phase of cell cycle, indicating that this subset of immature B cell is more activated than the T1 B cell subset and can develop into phenotypically mature follicular B cells after 1-2 days in vivo (Carsetti et al, 1995) and in vitro (Petro et al, 2002). T2 cells also actively proliferate in response to Baff (Batten et al, 2000).

T2 B cells characterised by $CD21^{high}$ $CD24^{high}$ generate proliferative, antiapoptotic and differentiation signals in response to BCR engagement, indicating that BCR signaling is very likely playing a critical role in T2 B cell survival and maturation, and these cells are a target for antigen driven positive selection (Su et al, 2002). Hardy's group do not show that T2 cells proliferate upon BCR engagement, this difference may be because they characterised their T2 subpopulation using alternative cell markers, namely IgM^{high} $CD23^{pos}$ $AA4^{pos}$ (Allman et al, 2001).

Recent evidence has suggested that B cell development is dependent on endogenous antigens and that only a minority of immature B cells are selected for maturation (Allman et al, 1993, Hardy and Hayakawa, 2001 and

Cariappa and Pillai, 2002). Once the immature B cell has reached the spleen it can develop into follicular (FO) or marginal zone (MZ) B cells and this decision is thought to be governed by the strength of B cell receptor (BCR) signaling (Cariappa et al, 2001). Cells that receive a weak signal via the BCR differentiate into marginal zone B cells and cells that receive strong signals via the BCR are likely to become follicular B cells (Pillai et al, 2004).

Targeted deletions of BCR signaling components, including Bruton's tyrosine kinase (Btk), phospholipase Cγ2 (PLCγ2), or protein kinase β (PKCβ). Each deletion leads to a loss of BCR dependent nuclear factor-κB (NFκB) activation, a crucial pathway for mediating cell survival, suggesting that one key response initiated by BCR engagement in T2 cells is NFκB dependent survival signaling (Su et al, 2002, Petro et al, 2000, Bajpai et al, 2000, Saijo et al, 2002, Wang et al, 2000 and Leitges et al, 1996). Disruption of downstream BCR signaling components, including Btk, C-cell linker protein (BLNK), phosphoinositide 3-kinase (PI3K), PLCγ2, or VAV result in developmental arrest at the T2 to mature B cell development (Su et al, 2002, Pappu et al, 1999, Xu et al, 2000, Suzuki et al, 1999, Fruman et al, 1999, Wang et al, 2000 and Martin and Kearney 2000).

Su et al, have proposed a model for a dual signaling pathway controlling peripheral B cell survival and differentiation. This model delineates two major BCR dependent signals: (I) a Btk-PKCβ-NFκB pathway required for the survival and maintenance of splenic B cells and (II) a Btk

dependant/PKCβ independent maturation signal driving the differentiation of T2 cells into the follicular mature B cell pool (Su et al, 2004).

Emerging in vivo data suggests that BCR and TLR activation are required for the generation of adaptive (FO) versus innate (MZ) mature B cells, respectively. In the presence of BCR engagement T2 cells are positively selected to become follicular B cell and if TLR specific bacterial ligands are also present, the generation of FO B cells are greatly enhanced. In the absence of strong BCR signals, T2 B cells have two fates: (I) In the presence of microbial products and TLR activation, T2 B cells differentiate into MZ B cells. (II) In the absence of both BCR and TLR stimulation, T2 B cells are likely to undergo cell death by neglect (Wei et al, 2004).

Baff (BLyS, TALL-1, THANK, z-TNF-4 and TNFSF20) B cell activating factor of the TNF family, plays a dominant lineage specific role in the selection and homeostatic control of the peripheral B cell pool, enhancing B cell survival. Baff is generated by monocytes, macrophages, neutrophils and dendritic cells and can be induced by a variety of inflammatory cytokines. Baff interacts with three receptors (BCMA, TACI, BR3/Bcmd/BAFF-R) found on B cells. Baff is thought to control the maturation of B cells, specially the development from immature to mature B cells. Baff deficient mice have normal numbers of T1 B cells, indicating that T1 cells do not depend on Baff for survival, but T2 and mature B cells in the periphery need Baff for survival (Schiemann et al, 2001). Baff-R deficient mice have a very similar phenotype to Baff deficient mice indicating that this

receptor is a dominant mediator of quiescent peripheral B cell survival (Thompson et al, 2001). Overexpression of Baff causes B cell hyperplasia, hyperglobulinaemia, splenomegaly, adenopathy, production of autoantibodies, glomerolonephritis, and expanded T2 and MZ B cells (Gross et al, 2000, Mackay et al, 1999 and Khare et al, 2000). Baff dictates the lifespan of B cells, thereby mediating homeostatic control of the peripheral B cell pool.

Recently, Pillai (Figure 6) suggested that there are two distinct types of transitional B cells and these cells can develop into follicular or marginal zone B cells. A specific "transitional follicular B cell precursor" (T2-FP), expressing IgM^{high} IgD^{high} $CD21^{intermediate}$ $CD1d^{low}$, these cells can be distinguished functionally from "transitional marginal zone B cell precursor" (T2-MZP), expressing IgM^{high} IgD^{high} $CD21^{high}$ $CD1d^{high}$ (Pillai et al, 2004). T2-MZP B cells are lost in mutant mice which do not make marginal zone B cells such as Aiolos and Notch-2, indicating that these cells are probably the MZ precursors (Cariappa et al, 2001 and Saito et al, 2003).

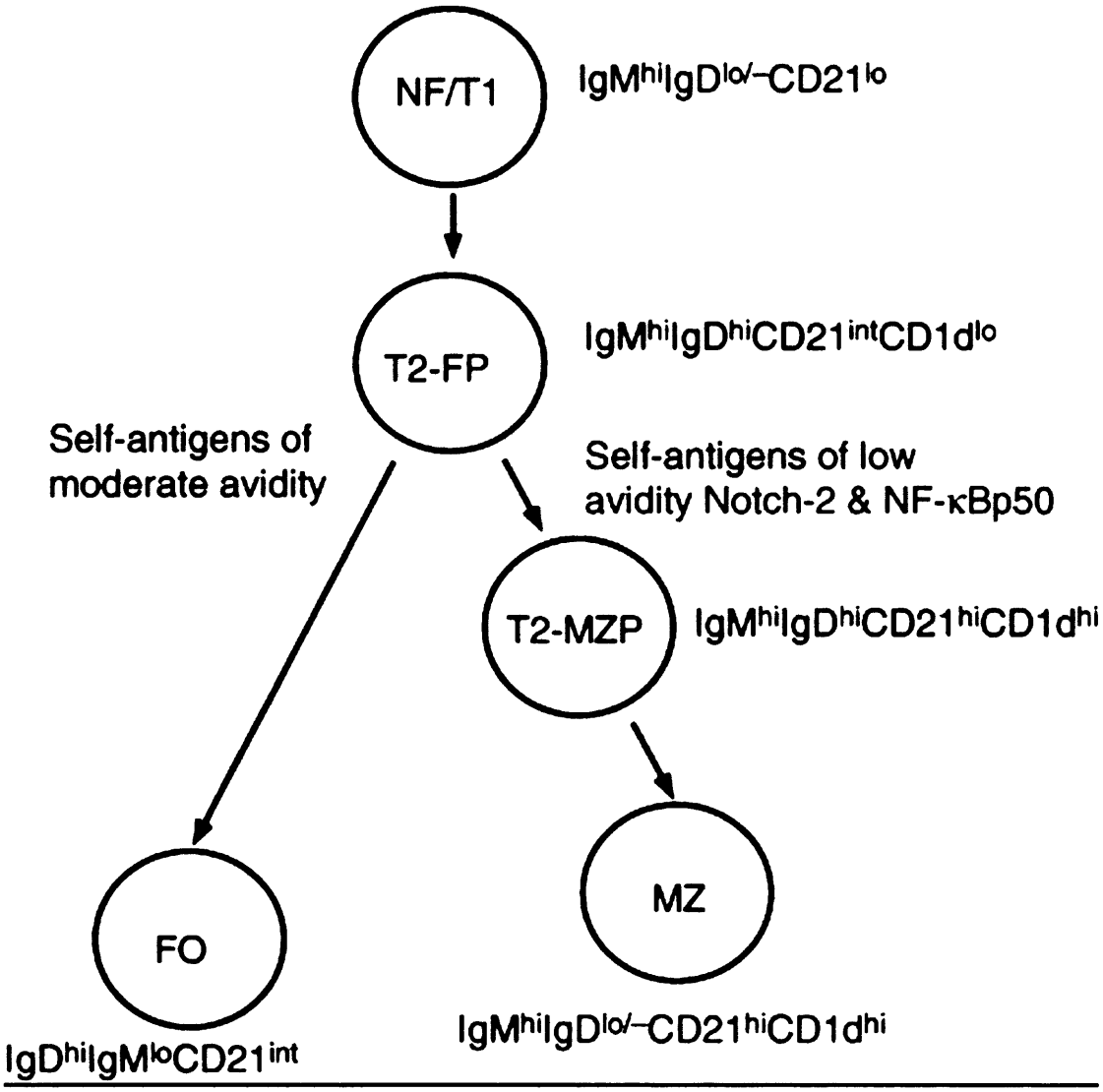

Figure 6. The follicular versus marginal zone B-lymphocyte cell-fate decision. Relatively strong (B-cell receptor) BCR signals drive FO B-cell differentiation, whereas weaker BCR signals are permissive for the development of MZ B cells via an intermediate MZP cell. MZ B-cell development is facilitated by Notch-2 and requires NF-Bp50. NF, newly formed B cell (equivalent to T1 cells); FP, follicular precursor B cells (a subset of B cells sometimes categorized as T2 cells); MZP, marginal zone precursor B cells (also called T2 cells); FO, follicular B cells; and MZ, MZ B cells (Pillai et al, 2004).

Follicular B cells

Developing B cells that receive relatively strong signals via the BCR (i.e. cells that recognise self-antigens with moderately high avidity) can readily commit to a mature follicular B cell fate (Pillai et al, 2004). Mature B cells are relatively long lived with a half-life of around 2-3 months (Hao and Rajewsky, 2001) and can be characterized by $B220^{high}$ CD19 IgM^{low} IgD^{high} $CD21^{intermediate}$ $CD23^{high}$ $CD24^{low}$

Follicular B cells reside primarily in the follicles of the splenic white pulp and recirculate to the lymph nodes and bone marrow. They are referred to as naïve, mature conventional B cells, they respond to antigen stimulation, and in the presence of costimulation by helper T cells participate in germinal center reaction. Within the germinal center activated follicular B cells proliferate and undergo somatic hypermutation, affinity maturation and isotype switching. These activated follicular B cells serve as antigen presenting cells, promoting T cell activation and further differentiate into highly specialized effector cells including plasma and memory B cells. Thus, the key B cell lineage outcome of germinal center reaction is an adaptive immune response characterised by the generation of high affinity, isotype switched, antigen specific antibodies (McLenann et al, 1997).

Marginal zone B cells

Marginal zone (MZ) B cells represent a distinct population of apparently "naïve" B cells with partially activated phenotype. They are sparse at birth, and mainly derived from hematopoietic stem cells of fetal liver origin. These cells are self-renewing and extremely long-lived, apparently surviving as long as the host (Hao and Rajewsky, 2001). In the spleen, they are exclusively located in the periphery of the periarteriolar lymphoid sheath (PALS), at the border of white and red pulp (Oliver et al, 1999 and Dammers et al, 1999).

MZ B cells are among the first population of cells to "see" blood-borne antigens and play an important role in host defence against bacterial pathogens (Martin et al, 2001a). These responses are primarily directed against T-independent multivalent antigens, however recently it was shown that MZ B cells can participate in T-dependent responses as well. Activated MZ B cells are potent protein antigens presenters to CD4 T cells and have the ability to induce antigen specific T cell clonal expansion and production of cytokines (Attanavanich et al, 2004).

They can be distinguished from other B cells by cell membrane markers. MZ B cells are B220 CD19 IgM^{high} IgD^{low} $CD21^{high}$ $CD23^{neg}$ $CD24^{high}$ and exhibit high levels of B7.1 and B7.2 indicative of previous antigenic experience (Oliver et al, 1999).

Mice that lack CD19, which enhances B cell signaling, have a selective reduction of MZ B cells (Martin and Kearney, 2000). Conversely,

mice that lack aiolos, a zinc finger transcription factor of the ikaros family expressed in B cells, which inhibits BCR signaling, have a reduced MZ (Cariappa et al, 2001). MZ B cells are also decreased in Lyn (cytoplasmic protein tyrosine kinase) deficient mice (Seo et al, 2001) and in mice in which the ITAM (immunoreceptor tyrosine-based activation motif) of Igα has been mutated (Kraus et al, 2001). Both Lyn and Igα are known to negatively regulate the BCR. Thus, MZ B cells are preferentially formed over follicular cells when there is a reduced signaling through the BCR.

Although BCR signaling plays a very important role in the differentiation of marginal zone B cell, other factors may influence it too, such as Notch. Notch 2 deficient B cells fail to generate MZ B cells. In mammals, the Notch family encodes four transmembrane receptor proteins (Notch 1-4) that influence cell fate decision by regulating transcription (Allman et al, 2002)

Notch-2, nuclear factor-κB, and other genes are essential for marginal zone B cell development, instructive signals delivered by the antigen receptor represent the primary force driving positive selection and lineage commitment in B lymphocytes (Pillai et al, 2004).

B1 B cells

B1 cells were first described based on the phenotype; B1 cells (fetal B cell development) expressing CD5+ and B2 or conventional B cells (generated in adult bone marrow) not expressing CD5. B1 phenotype B cells are further sub-divided into B1a (CD5+) and B1b (CD5-) (Hardy et al, 2001). Recent data reveal differences between B1 compared with B2 B cells development in terms of initial repertoire establishment and cellular selection. It appears that CD5 induction may be a consequence of positive selection in B1 development resulting in enrichment of auto-reactive B cells in this population (Hayakawa and Hardy, 2000). There is some suggestion that it is possible to generate auto-reactive "B1 like" CD5- cells during B2 development, possibly by deregulation of normal tolerance mechanisms (Cong et al, 1991).

B1 cells are the predominant B cell type in the peritoneal cavity, are infrequent in the spleen, and generally absent in the lymph nodes and bone marrow, whereas conventional B2 cells are the predominant B cells type in the spleen and lymph nodes. B-1 cells have characteristics of activated cells, they are larger and more granular. Their V_H and V_κ repertoires differ considerably from those of conventional B cells. High frequencies of B 1 B cells are polyreactive and autoreactive. These include B cells specific for single-stranded DNA, rheumatoid factor, phosphatidyl choline and also includes B cells specific for bacterial carbohydrate antigens and phosphoryl

choline, prompting B1 cells for T independent response to common bacterial antigens (Tatu et al, 1999).

B1 cell development and maintenance appear to depend on BCR signaling, because a reduction of B1 cell numbers occurs if there is insufficient BCR signaling or insufficient antigen stimulation. With the use of knock outs and transgenic mice it is possible to analyse this phenotype, although most of the studies were done in adult mice and the defect in signaling may act at several stages: from pre-B to mature B cell (Hardy et al, 2001).

Engagement of the BCR activates cytoplasmic protein tyrosine kinases such as Syk, Lyn, Blk and Bruton's tyrosine kinase (Btk), and can lead to a multitude of cellular responses, such as proliferation, activation, differentiation or cell death. It has been demonstrated that adaptor proteins play a major role in interfacing tyrosine kinase activation by lymphocyte antigen receptors with selective downstream signaling molecules. One such adaptor molecule termed BLNK, SLP-65 or BASH has been identified in B cells and is specifically involved in BCR signaling. BLNK can associate with Btk and also couple Syk and VAV, and is intimately associated with intracellular calcium mobilisation, which is essential for cell activation. Phosphoinositide 3-kinase (PI3-kinase) is responsible for the production of phosphatidylinositol-trisphosphate and participates in various signal transduction pathways (Xu et al, 2000). The hematopoietic protooncogene VAV proteins belong to the DBL family of RHO guanine nucleotide exchange

factors (GEFs) and are known to have an important role in regulating early events in receptor signaling (Tarakhovsky et al, 1995 and Zhang et al, 1995).

When mice are made deficient for different molecules that regulate signaling through BCR, the number of B1 cells are likely to be reduced. Various examples can be found in the literature, such as CD19 a BCR co-stimulatory transmembrane protein (Inaoki et al, 1997), the BLNK/SLP-65 adaptor protein (Xu et al, 2000), the Cr2 gene encoded CD21 molecule that complexes with CD19 (Carroll et al, 1998), VAV (Tarakhovsky et al, 1995 and Zhang et al, 1995), the PI3-kinase (Suzuki et al, 1999 and Fruman et al, 1999) and the Btk tyrosine kinase (Khan et al, 1995) knockout mice all have decreased numbers of CD5+B cells. Since most of these factors are known to play roles in BCR mediated activation, the decrease in CD5+ B cells have been attributed to disruption of activation–mediated upregulation of CD5 expression on B cells (Hardy and Hayakawa, 2001). Knockout cyclin D2 mice, show a defect on cell cycle progression and also have a decrease in CD5+ B cells (Solvason et al, 2000).

Transgenic mice with BCRs of restricted specificity show, depending on the specificity, increased numbers of CD5+ B cells; 3H9μ-TG, M54μ-TG 3-83μδ-TG, specific to H-2K^{k}D^k, have decreased numbers of CD5+ B cells, and VH11μ-TG, specific to phosphorylcholine (Chumley et al, 2000), VH12μ-TG also specific to phosphorylcholine, and VH3609μ-TG (Hayakawa et al, 1990) have their numbers of CD5+ B cells increased. This difference is

explained by the fact that peritoneal B1 B cells are positively selected by self-antigens.

BCR-mediated signaling is also controlled by negative regulators, such as the CD22 BCR adaptor molecule. The src family kinase, Lyn, based on its capacity to phosphorylate the inhibitory receptors FcRIIb or CD22. PD-1, an immunoglobulin superfamily member with an ITIM motif. SHP-1, a protein-tyrosine phosphatase with two src-homology 2 domains. FcRIIb, CD22 and PD-1 can recruit SHP-1 protein tyrosine phosphatase that can play a major role in negative signalling.

In mice with strengthened BCR signal there is an increased number of CD5+ cells. It was observed on SHP-1 mutant (me) (Sidman et al, 1986) and CD19-TG (Sato et al, 1996b). CD22, lyn and PD-1 knockout mice all have an increased number of B1b B cells (Chan et al, 1997, Nishizumi et al, 1995 and Wang et al, 1996). The increase in B-1b cells in CD22-, lyn-, or PD-1-deficient adult animals might be due to a breakdown in tolerance during B-2 development, since adult bone marrow cells can generate B-1b type cells (Hardy and Hayakawa, 2001).

Similarities between MZ and B1 B cells

Alterations in some molecules that affect B cell signaling result in parallel changes in MZ and B1 B cells. B cells with diminished BCR signaling, including CD45, CD19, CD21 and PI3Kδ deficient mice, lack MZ and B1 B cells (Rickert et al, 1995, Sato et al, 1996b, Ahearn et al, 1996, Martin and Kearney, 2000 and Clayton et al, 2002), and mice with enhanced B cell signaling such as mice lacking PTEN, a negative regulator of PI3K are characterised by enhanced numbers of MZ and B1 B cells (Anzelon et al, 2003). Other examples demonstrate that these two B cell subsets are not always regulated in tandem such as CD22 deficient mice where B1 cells are increased but MZ cells are reduced (O'Keefe et al, 1996, Sato et al, 1996a and Samardzic et al, 2002), though one group showed no change in B1 cell numbers (Otibody et al, 1996).

Marginal zone (MZ) and B1 B cells have been implicated in the rapid, early response to infection, T-independent response, in autoimmunity and lymphogenesis (Hayakawa and Hardy, 2000, Martin and Kearney, 2001 and Morse et al, 2001). For example, NZB/W lupus prone mice have an elevated proportion of B1 and MZ B cells (Wither et al, 2000).

These two population are also targets of positive selection, this viewpoint was demonstrated by Cyster and Goodnow, who showed that CD45 deficient cells can only enter the long lived B cell pool if exposed to well defined, transgene encoded self-antigens (Cyster et al, 1996). MZ and B1 B cells have a repertoire biased by positive selection, where MZ are

enriched with B cells expressing the phosphoryl choline binding idiotype M167 or fetal associated idiotype 81X, but not B cells specific for hen lysozyme (Martin and Kearney, 2000) and B1 B cells are enriched with cells specific for single-stranded DNA, rheumatoid factor and phosphatidyl choline (Tatu et al, 1999).

Table 4 shows similarities and differences between MZ and B1 B cell, as well as T1, T2 and follicular B cells and table 5 shows the effect of mutations in molecules that are important for the development of B cells.

Table 4. Comparison of phenotype and function of B cell subsets.

Phenotype	B1 IgM^{high} IgD^{low} $CD21^{low}$ $CD23^{neg}$ CD5+	T1 IgM^{high} IgD^{low} $CD21^{low}$ $CD23^{neg}$	T2 IgM^{high} IgD^{high} $CD21^{high}$ $CD23^{pos}$	MZ IgM^{high} IgD^{low} $CD21^{high}$ $CD23^{neg}$	FO IgM^{low} IgD^{high} $CD21^{inter}$ $CD23^{high}$
Half-life	Long	Short	Short	Long	Long
Blood circulation	+	++	-	-	+
Lymph circulation	-	-	-	-	+
TI responses	+++	-	+++	+++	+
TD responses	+/-	-	-	+	+
Ag presentation in vitro	+++	-	+	+++	+
Time to peak cell cycle	Short	Long	Short	Short	Long
Apoptosis after anti-IgM	-	+++	+	+++	+
BCR positive selection	++	-	++	++	+/-
Proliferation					
LPS	+++	-	+	++	-
LPS+IgM	NA	-	+++	+++	+++
CD40	++	+	+	++	+
IgM	-	-	+/-	-	+
IgM+CD40	++	+	+	++	++

Adapted from Martin and Kearney, 2002 and Su et al, 2004

Table 5. Mutations in molecules that affect B cell development

Mutation	***Observations***	***References***
Aiolos	Reduction of MZ and B1 B cells	Cariappa et al, 2001
Baff	Developmental arrest at the T2 to mature B cell development	Schiemann et al, 2001
Baff-R	Developmental arrest at the T2 to mature B cell development	Thompson et al, 2001
BLNK	Reduction of B1 B cells	Xu et al, 2000, Pappu et al, 1999
Btk	Developmental arrest at the T2 to mature B cell development	Martin and Kearney, 2000, Petro et al, 2000, Bajpai et al, 2000
CD19	Reduction of MZ and B1 B cells	Martin and Kearney, 2000, Inaoki et al, 1997
CD21	Reduction of MZ and B1 B cells	Carroll et al, 1998, Cariappa et al, 2001
CD22	Increased B1 B cells and MZ B cells are reduced	Chan et al, 1997, O'Keefe et al, 1996, Sato et al, 1996a, Samardzic et al, 2002
CD45	Reduction of MZ and B1 B cells	Cyster et al, 1996
Cyclin D2	Reduction of B1 B cells	Solvason et al, 2000
Igα	Developmental arrest at the T1 stage of B cell development	Torres et al, 1996
Igα with mutations in the ITAM	Reduction of MZ B cells	Kraus et al, 2001
Lyn	Reduction of MZ B cells and increased B1 B cells	Seo et al, 2001, Nishizumi et al, 1995
Notch 2	Fail to generate MZ B cells	Allman et al, 2002
PD-1	Increased B1 B cells	Wang et al, 1996
PI3K	Reduction of MZ and B1 B cells	Suzuki et al, 1999, Fruman et al, 1999
PKCβ	Developmental arrest at the T2 to mature B cell development	Leitges et al, 1996, Saijo et al, 2002
PLCγ2	Developmental arrest at the T2 to mature B cell development	Wang et al, 2000
PTEN	Increased B1 and MZ B cells	Anzelon et al, 2003
SHP-1	Increased B1 B cells	Sidman et al, 1986
VAV	Reduction of MZ and B1 B cells	Tarakhovsky et al 1995, Zhang et al, 1995

T independent response

Although peptide-specific helper T cells are required for B cell responses to protein antigens, many microbial constituents, such as bacterial polysaccharides, can induce antibody production in the absence of helper T cells. These microbial antigens are known as thymus-independent or TI antigens. The signal required to activate antibody production to TI antigens is either provided directly by recognition of a common microbial constituent, or by a non thymus-derived accessory cell in conjunction with massive cross-linking of B cell receptors, which would occur when a B cell binds repeating epitopes on the bacterial cell. Thymus independent antibody responses provide some protection against some extracellular bacteria.

TI-1 antigens possess an intrinsic activity that can directly induce B-cell division. At high concentration, these molecules cause proliferation and differentiation of most B cells regardless of their antigen specificity. An example of a B-cell mitogen and TI-1 antigen is LPS.

Another class of thymus-independent antigens consists of molecules such as bacterial capsular polysaccharides that have highly repetitive structures. These thymus-independent antigens (TI-2 antigens) contain no intrinsic B cell stimulating activity. TI-1 antigens can activate both immature and mature B cells, TI-2 antigens can activate only mature B cells.

Responses to several TI-2 antigens are common among B-1 cells and marginal zone B cells.

TI-2 antigens act by extensively cross-linking the B-cell receptors of mature B cells specific for the antigen. Excessive receptor cross-linking makes immature and mature B cells unresponsive or anergic (Janeway et al, 2001 and Shih et al, 2002).

IgM deficient mice

Natural serum IgM provides the first defence against infection and accelerates the development of the primary immune response. Mice deficient in serum IgM were more susceptible to peritonitis induced by cecal ligation and puncture. This susceptibility was due to the reduced level of TNFα and a decrease in neutrophil recruitment causing an increase of bacterial load in the peritoneum (Boes et al, 1998b). IgM deficient mice also show significantly reduced viral clearance and survival when infected with influenza virus (Baumgarth et al, 2000). It is clear that serum IgM is an important source of natural antibody and act as the first barrier to viral and bacterial infection.

A mutant mouse that does not secrete IgM, but still expresses membrane bound IgM, was made by deleting the Cμ secretory tailpiece and the μS polyadenylation site by Lox/Cre gene mediated gene targeting. These mice have normal levels of all Ig isotypes, apart from IgM, which is

absent in the serum. B cell proliferation is normal in response to LPS or IL4 and anti-IgM. They show an elevated number of B1 B cells in the peritoneum. The T cell independent response to NP-Ficoll is increased, but reduced to T dependent antigens such as phOx-CSA (Ehrenstein et al, 1998). These mice have an increased predisposition to develop IgG anti-DNA antibodies and renal deposition of IgG and complement with age. When immunised with LPS, IgM deficient mice show an augmented anti-DNA response (Ehrenstein et al, 2000). This suggests that the lack of serum IgM may influence the development of autoimmunity.

Another group also made a secretory IgM deficient mouse by targeting the μ_s exon and its three downstream polyadenylation sites and then replacing it by a cDNA fragment encoding the Cμ4 and μ_m exons. Their findings were very similar, but elevated levels of IgG2a, IgG3, and IgA were observed in the mutant mice at an early age (Boes et al, 1998a).

Boes and colleagues also demonstrated the development of autoimmunity in IgM deficient mice. They crossed IgM mutant mice onto a lupus-prone lymphoproliferative (*lpr*) mice bred into a MRL background. MRL/lpr mice spontaneously develop autoimmunity characterised by high levels of anti-DNA antibody and glomerulonephritis (Theofilopoulos and Dixon, 1985). *Lpr* is a mutation in the Fas (CD95) gene, a member of the tumor necrosis factor receptor family, and binding of the ligand, Fas-L, which triggers apoptosis in many cell types. The failure of apoptosis by *lpr* mutation results in lymphoproliferative disease, including the emergence of

oligoclonally expanded, autoreactive B cells bearing somatically mutated IgGs (Shlomchik et al, 1987 and Watanabe-Fukunaga et al, 1992).

In lupus-prone mice, the absence of secreted IgM accelerated the development of IgG autoantibodies and glomerolonephritis, and the mice succumbed to disease at an earlier age (Boes et al, 2000).

Another approach was used by (Chan et al, 1999) with MRL*/pr* mice expressing a mutant transgene encoding surface immunoglobulin, but which did not permit the secretion of circulating Ig. These mice develop nephritis, characterised by cellular infiltration within the kidney, indicating that B cells without the production of antibodies, act as either antigen-presenting cells for autoreactive T cells, and/or by contributing to the inflammation site, exert a pathogenic role.

To test the role of Fas in the elimination of defective B cells, Melamed et al, 2000, introduced the *lpr* mutation to mice lacking the IgM transmembrane tail exons, in which B cell development is blocked at the pro-B stage. They showed that these mice developed significantly enhanced autoimmunity and autoantibody production mainly because the absence of functional Fas allowed for defective B cells to be generated and selected to participate in the autoimmune process.

The influence of genetic background in autoimmunity

Autoimmune diseases are complex disorders characterized by adaptive immune response that are inappropriately directed against self tissues. Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by elevated levels of IgG antinuclear antibodies and the development of an immune-complex mediated glomerulunephritis (Kotzin 1996). The genetic background seems to play an important role in the development of autoimmunity.

New Zealand black (NZB) mice are one of the best known models of lupus, it is not sex linked and they spontaneously develop haemolytic anaemia, glomerulonephritis and lymphomas. NZB when crossed with NZW (New Zealand white) mice have a progeny called NZB/W (New Zealand black white) mice, where the females spontaneously develop a lupus like disease characterized by severe immuno-complex nephritis (Theofilopoulos and Dixon 1985).

Female MRL/*lpr* mice develop a fatal immune-complex glomerulunephritis at an early age, and both sexes develop a very severe lymphoproliferation (*lpr)*. MRL+/+ develop a similar, but milder and with a later onset form of lupus. In contrast the *lpr* gene in other strains such as C57BL/6 leads only to autoantibody production (Izui et al, 1984).

The BXSB is a recombinant inbred strain resulting from crossing C57BL/6J females with SB/Ie males. It is an unique model as the males

develop the most severe form of the disease, which is characterised by haemolytic anaemia, lymphoadenopathy and glomerulonephritis (Izui et al, 1995).

Genetic mapping has identified multiple intervals associated with disease susceptibility. The majority of the intervals detected are strain specific, confirming the genetic complexity of the disease and indicating the presence of extensive heterogeneity in the genes contributing to the pathogenesis of the disease. However, loci on distal Chromosome 1 have been found on both NZ and BXSB model indicating that some susceptibility may be shared amongst lupus prone strains (Wakeland et al, 2001).

HYPOTHESIS

Serum IgM has multiple functions in the immune system. It is proposed that serum IgM by virtue of its polyreactivity can boost signals delivered via the BCR. It is thought that the strength of BCR signalling can regulate the selection of B cell subsets, and thus natural IgM may play a role by forming complexes with (auto)antigens to make them more immunogenic. The hypothesis to be tested is that lack of serum IgM alters the regulation/selection of B cell subsets.

IgM has also been shown to be important in autoimmunity and it is proposed that lack of serum IgM would lead to increased autoimmunity in an autoimmune predisposed mouse strain.

AIMS

1) To characterise the changes in B cell subsets in mice deficient in serum IgM.
2) To characterise the cause of the changes in B cell subsets in mice deficient in serum IgM.
3) To investigate the mechanisms that underlie the alterations in the B cell subsets by reconstituting serum IgM deficient mice with polyclonal or monoclonal IgM.
4) To analyse the T-independent immune response in serum IgM deficient mice and determine the serological and cellular responses when different forms of IgM are administered at the same time as the antigen.
5) To investigate further the relationship between deficiency of serum IgM and autoimmunity by intercrossing the serum IgM deficient mice with the mild autoimmune strain BL/6 *lpr* mice.

MATERIALS AND METHODS

MATERIALS AND METHODS

Mice

Breeding of Sμ- mice

The generation of Sμ- mice has been described previously (Ehrenstein et. al, 1998). Mice were backcrossed onto C57BL/6 for 6 generations and kept under specific-pathogen-free conditions. Sμ- and litter matched controls mice used for the experiments were 3-12 month old. PCR and ELISA were performed to ensure that IgM was not being secreted.

Generation and breeding of Vμ1s mice

The Vμ1 construct (kind gift from Dr Michael Neuberger, Laboratory of Molecular Biology, Cambridge, UK) encodes a VH (*a* allotype) gene, which binds to (4-hydroxy-3-nitrophenylacetyl - NP), under control of a VH promoter (Sitia et. al, 1990). It was modified by restriction enzyme digestion (Cla I site upstream of the VH promoter to Kpn which is 1Kb 3' of the μs poly A site but 5' of the μM1 splice acceptor site). The membrane exon of the transgene was therefore deleted but the secretory exon remained intact. The resulting 11kb construct Vμ1s (Figure 7A) was used for pronuclear injection and transgenic mice were generated by standard techniques (UCL-Gene Targeting and Transgenic Core Facility). The mice were tested for the presence of the transgene by PCR, the expression of IgM in the serum by

ELISA and FACs was used to ensure that there was no B cell membrane transgene expression. These mice were backcrossed 6 times onto C57BL/6 mice and subsequently onto Sμ- mice.

Breeding of Sμ- onto C57BL/6 *lpr* mice

Serum IgM deficient mice were intercrossed onto a C57BL/6 mice homozygous for the *lpr* mutation of Fas (CD95). Mice were kept under specific-pathogen-free conditions. Sμ-, *lpr,* Sμ- *lpr* and litter matched controls mice were 3-12 month old. PCR was performed to ensure that the mice were Fas mutant (Singer and Abbas 1994).

Genotyping

Preparation of genomic DNA

Tail tip biopsies of mice were placed in 500μl of tail buffer (100mM Tris pH8.5, 5mM EDTA, 0.2% SDS and 200mM NaCl-Sigma) plus 35μl of 10mg/ml proteinase K (Sigma), and the tissue was digested over night at 55^0C. The digested tissue was extracted using the same volume of equilibrated phenol (USB) and the aqueous supernatant was again extracted using the same volume of 24:1 of chloroform and isoamylalcohol (BDH). The DNA was precipitated with 0.8 volumes of isopropanol (BDH), the precipitated DNA was washed with 70% ethanol (BDH) and air dried before being redissolved in 200ul of purified water (Sigma).

PCR for genotyping Sμ-, Vμ1s and *lpr* mice

PCR was routinely carried out using PCR thermalcycler (Biometra) in accordance with the manufactures' instructions. Genotyping was performed using 1 μl of genomic DNA from tail tip biopsies. Taq polymerase (Promega) was used for genotyping Vμ1s and *lpr*, and TaqPlus Long (Stratagene) was used for Sμ- according to the manufactures' instructions. Oligonucleotides were produced by MWG and used at concentration of 100ng/μl. The fragments were analysed on agarose (Sigma) gels containing ethidium bromide (Sigma).

Primers used for genotyping

Sμ- mice

5' cct tac ttc ctg aag gac tcg g

5' cta gag gca tct ctc cct gtc

Vμ1s mice

5' tat gta tcc tgc tca tga at

5' ttt taa gga ctc acc tga gg

***lpr* mice**

5' aat aat tgt gct tcg tca g

5' tag aaa ggt gca cgg gtg tg

5' caa atc tag gca tta aca gtg

Injections and immunisation

Injection of IgM polyclonal and monoclonal

Sμ- and litter matched controls were injected i.p. 3x a week for 2 weeks with 200μg/mouse (concentration of serum IgM in wild type mice) of a purified monoclonal (either TEPC-183, unknown specificity (here designated monoclonal A) or MOPC-104E, specific for -1,3-glucose (here designated monoclonal B) from Sigma or a polyclonal IgM antibody from Rockland. The antibodies were chosen according to availability in the market.

Immunisation with PC-Ficoll

The hapten PC-Ficoll was chosen because it does not bind to the transgene Vμ1s like NP, and specifically stimulates marginal zone B cells and not B1 B cells. To determine the T independent response against phosphorylcholine hydroxyphenylactic acid (PC)-Ficoll (Bioresearch Technologies), mice were immunised i.p. with 10μg of PC-Ficoll in PBS. Sμ-, Vμ1s, Sμ- mono, Sμ- poly and litter matched controls were bled via the tail vein on day 0, 7 and day 14 after immunisation.

Immunoassays

ELISA for serum IgM

The titres of IgM in the serum of Sμ-, Vμ1s, Sμ- mono, Sμ- poly and litter matched control were performed on NUNC maxisorp plates. The wells were coated for 2 hours with 2μg/ml of purified anti-mouse IgM (II/41) (Pharmingen) diluted in PBS (1L of 10x PBS: KCl 2g, KH_2PO_4 2g, Na_2HPO_4 11.48g, NaCl 80g - Sigma) and blocked overnight at 4°C with PBS/2%BSA (Serum albumin bovine – Sigma). 1μl of serum was added in serial dilutions. 2μg/ml of biotinylated rat anti-mouse IgM (R6-60.2) was used when measuring total IgM, when measuring the Vμ1s transgene and the amount of IgM after the mono or poly injections IgM*a* (DS-1) (Pharmingen) was used as secondary antibody. The bound antibodies were detected using streptavidin-horseradish peroxidase (HRP) from Dako and developed by ABTS (Sigma) in 20ml H_2O, 0.5ml 1M Na Citrat, 0.5ml 1M Citric Acid and 4μl H_2O_2. PBS/0.5% tween 20 (Sigma) was used to wash the plates between each step. Known concentrations of purified IgM (MOPC-104E Sigma) antibody was used to calculate the dilution curves. Plates were read at 409 nm (Anthos HTII).

ELISA anti-phosphorylcholine (PC) Ficoll

ELISA was performed to measure IgG_3, (IgM and IgG_3 are the main antibodies produced in T-independent responses, in the absence of IgM,

IgG_3 was measured) anti-phosphorylcholine (PC) Ficoll on NUNC maxisorp plates. The wells were coated for 2 hours with 10μg/ml of PC-BSA (Bioresearch Technologies) diluted in PBS and blocked overnight at 4°C with PBS/2%BSA. 1μl of serum, from mice bled on day 0, 7 and 14 after IgM injection, was added in serial dilutions. 2μg/ml of biotinylated rat anti-mouse IgG_3 (R40-82) from Pharmingen was used as the secondary antibody. The bound antibodies were detected using streptavidin-horseradish peroxidase (HRP) from Dako and developed by ABTS (Sigma). Plates were read at 409 nm (Anthos HTII).

The monoclonal and polyclonal IgM antibodies that were used for injections were tested against phosphorylcholine to detect specific binding using biotinylated rat anti-mouse IgM (R6-60.2) from Pharmingen as secondary antibody.

ELISA anti-dsDNA

Sμ-, *lpr,* Sμ- *lpr* and litter matched controls were bled via the tail vein at intervals of 4 weeks for 12 months to follow the development of IgG anti-double stranded (dsDNA) antibodies.

NUNC maxisorp plates were pre coated for 2 hours with 2.5μg/ml of poly-L-Lysine (Sigma) diluted in distilled water and coated for 2 hours with 0.1μg/ml of dsDNA diluted in PBS and blocked overnight at 4°C with PBS/2%BSA. 1μl of serum was added in serial dilutions. 2μg/ml of goat anti-mouse IgG conjugated to alkaline phosphatase (Southern

Biotechnology Inc.) was used. The binding of antibodies to dsDNA was detected using phosphatase substrate PNPP (Sigma) dissolved in bicarbonate buffer pH 9.4 (1L: Sodium carbonate 0.8g, Sodium bicarbonate 1.55g - Sigma). Plates were read at 409 nm (Anthos HTII).

The serum of a MRL/*pr* mouse known to have positive titres of IgG anti-dsDNA was used as the positive control and given value 1 Unit in all plates.

The monoclonal and polyclonal IgM antibodies that were used for injections were tested against dsDNA to detect specific binding as described above using goat anti-mouse IgM conjugated to alkaline phosphatase (Southern Biotechnology Inc.) as secondary antibody.

ELISA anti-histones

The monoclonal and polyclonal IgM antibodies that were used for injections were tested against histones to detect specific binding. NUNC maxisorp plates were coated over night at 4 °C with 10μg/ml of histone type IIS (Sigma) diluted in PBS and blocked for 2 hours at room temperature with PBS/2%BSA, and any DNA contaminating the histone was removed by treatment with DNAse 1 (Sigma). 1μl of serum was added in serial dilutions. 2μg/ml of goat anti-mouse IgM conjugated to alkaline phosphatase (Southern Biotechnology Inc.) was used. The binding of antibodies to dsDNA was detected using phosphatase substrate PNPP (Sigma) dissolved in bicarbonate buffer pH 9.4. Plates were read at 409 nm (Anthos HTII).

ELISA anti- myeloperoxidase (MPO)

The monoclonal and polyclonal IgM antibodies that were used for injections were tested against myeloperoxidase to detect specific binding. The myeloperoxidase ELISA was carried out using a Captia myeloperoxidase ELISA kit (Trinity Biotech, Bray, Ireland) in accordance with the manufactures' instructions.

Cell isolation and cell culture

Sμ-, Vμ1s, Sμ- mono, Sμ- poly, *lpr,* Sμ- *lpr* and litter matched control peritoneal cells were isolated by flushing the peritoneal cavity with 2 ml of PBS supplemented with 0.2% fetal calf serum FCS (Gibco). Splenic and lymph node cell suspensions were prepared using mechanical disruption of the tissue and passed through a 70 μm nylon mesh. Cell suspensions were made following lyses of erythrocytes using Pharm Lyse (Pharmingen).

For some experiments splenocytes were cultured in B cell medium (RPMI, 5% FCS, 2nM glutamine, 1mM pyruvate, 1x MEM non essential amino acids, 10 units penicillin and streptomycin, 0.1mg/ml gentamycin and 50uM 2ME - Gibco) alone or with 10ug/ml of F(ab')2 goat anti-mouse IgM (Southern Biotechnology Inc) at 37 ^{0}C 5% CO_2 for 2 or 24 hours, before the cells were stained for surface markers.

Flow cytometric analyses

Flow cytometric analyses were performed on Sμ-, Vμ1s, Sμ- mono, Sμ- poly, *lpr*, Sμ- *lpr* and litter matched controls to determine the expression of cell surface markers. $1x10^6$ splenic, lymph node or peritoneal cells were stained for 20 minutes with 0.5μg of the following conjugated monoclonal anti-mouse antibodies (Pharmingen):

Biotinylated	Fluorescein Isothiocyanate (FITC)	R-Phycoerythrin (R-PE)
CD23 (B3B4)	IgM (R6-60.2)	CD45R/B220 (RA3-6B2)
CD5 (53-7.3)	CD3 (17A2)	IgD (11-26c.2a)
IgM*a* (DS-1)	CD21/CD35 (7G6)	IgM (R6-60.2)
IgM*b* (AF6-78)		CD19 (1D3)
IgD (11-26c.2a)		

The cells were then washed with PBS/0.2%FCS and Cy5-conjugated to streptavidin (Dako) was added to biotinylated monoclonal antibodies followed by a further 20 minutes incubation and appropriate washes. Stained cells were analysed on FACs scan or BD-LSR and files were plotted using CELLQuest software both from BD Biosciences.

Immunoflourescence staining of tissue sections

Standard immunoflourescence staining procedures were used. OCT-embedded, snap-frozen spleens were sectioned at 8μm (Leica CM 1900), air-dried and acetone-fixed at 20 ^{0}C. The sections were blocked for nonspecific binding with PBS/2%FCS, stained with 10μg/ml of FITC conjugated anti-mouse IgM (R6-60.2) and biotinylated anti-mouse IgD (11-26c.2a) (Pharmingen), incubated for 2 hours at 4 ^{0}C in the dark followed by washes with PBS and Texas Red-conjugated to streptavidin (Dako) was added to biotinylated monoclonal antibodies and incubated for 2 hours at 4^{0}C in the dark followed by washes with PBS. Sections were mounted with VECTASHIELD (Vector). Sections were viewed (10x) with a Zeiss Axiovert 100 TV fluorescence microscope equipped with filters of 488 and 568 nm for FITC and Texas Red respectively. Images were acquired and processed using cofocal assistant 4.02 (Todd Clark Brelje) and Abode Photoshop 4.0 (Adobe).

In vivo 5-bromo-2'-deoxyuridine (BrdU) incorporation

In vivo 5-bromo-2'-deoxyuridine (BrdU) incorporation was used to access the turnover rate of B lymphocytes in the spleen and peritoneal cavity of four month old Sμ- and litter matched control. Mice had BrdU (BD biosciences) through drinking water diluted to 0.8mg/ml every 12 h for 3 or 7

days. Spleen and peritoneal cells were stained for surface expression of CD23, CD21, IgM, IgD, CD5 and B220 using standard FACS techniques as described above, followed by fixation, permeabilisation, treatment of cells with DNase, intracellular staining for BrdU and flow cytometric analysis all performed according to the manufactures' instructions (BrdU Flow Kit-Pharmingen). Cells were analysed on BD-LSR, and files were plotted using CELLQuest software both from BD Biosciences.

Apoptosis measured by terminal dUTP nucleotide end labelling (TUNEL)

The incorporation of FITC-dUTP into DNA strand breaks by the terminal dUTP nucleotide end labelling (TUNEL) method was used to determine apoptosis. Sμ- and litter matched control 4-month-old mice were used for these experiments. Spleen and peritoneal cells were stained for surface expression of CD23, CD21, IgM, IgD, CD5 and B220 using standard FACS techniques as described above, followed by fixation, permeabilisation, labelling reaction with TUNEL and flow cytometric analysis was performed according to the manufactures' instructions (In Situ Cell Death Detection Kit, Fluorescein-Roche). Cells were analysed on BD-LSR, and files were plotted using CELLQuest software both from BD Biosciences.

Measurement of intracellular calcium flux mobilisation

Spleen and peritoneal cells were stained for surface expression of CD23, CD21, CD5 and B220 using standard FACS techniques as described above, cells were loaded with Indo-1AM (Molecular Probes) for 1 hour at 37°C. Cells were washed twice, resuspended at a concentration of 2 x 10^6 cells/ml in RPMI and 5% FCS, and stimulated with 1μg/ml up to 30μg/ml of F(ab')2 goat anti-mouse IgM (Southern Biotechnology) for few seconds. Calcium flux was measured by calculating the ratio of calcium bound indo-1 (FL5) and unbound (FL4) versus time using a BD-LSR (BD Biosciences), which allows for 6 parameter flow cytometry including real time analysis of changes in UV fluorescence on gated populations. Data analysis was performed using CellQuest (BD Biosciences) and FloJo (Tree Star) software.

Statistical analyses

Means were compared by the Student's T Test and differences were considered significant when p value was < 0.05.

RESULTS

RESULTS

B cell development

Marginal zone B cells are expanded in serum IgM deficient (Sμ-) mice

Mature splenic B cells can be divided into marginal zone and follicular B cells and can be distinguished by surface markers using FACS. Marginal zone B cells are IgM^{high} IgD^{low} $CD21^{high}$ $CD23^{neg}$ and were expanded in 4-month-old Sμ- mice whereas follicular B cells were reduced (Figure 7A). The increase in MZ B cell numbers was 2.5-fold, the reduction in follicular B cells was less (a 25% reduction) based on proportion of absolute cell numbers for individual mice. Wild-type (WT) and Sμ- mice have the same number of splenic B220+ B lymphocytes (WT $1.27x10^7$ ± 0.26, Sμ- $1.12x10^7$± 0.17). In older mice (12 month old) the MZ:FO B cell ratio was increased in WT mice and the difference in MZ:FO B cell ratio between Sμ- and WT mice was less striking at this age (Figure 7B).

Spleen sections taken from naive 4 month old Sμ- mice stained with anti-IgM and anti-IgD demonstrated a larger IgM^{bright} IgD^{dull} MZ area on the periphery of the follicle compared with WT controls, further demonstrating the expansion in MZ B cells in Sμ- mice (Figure 8).

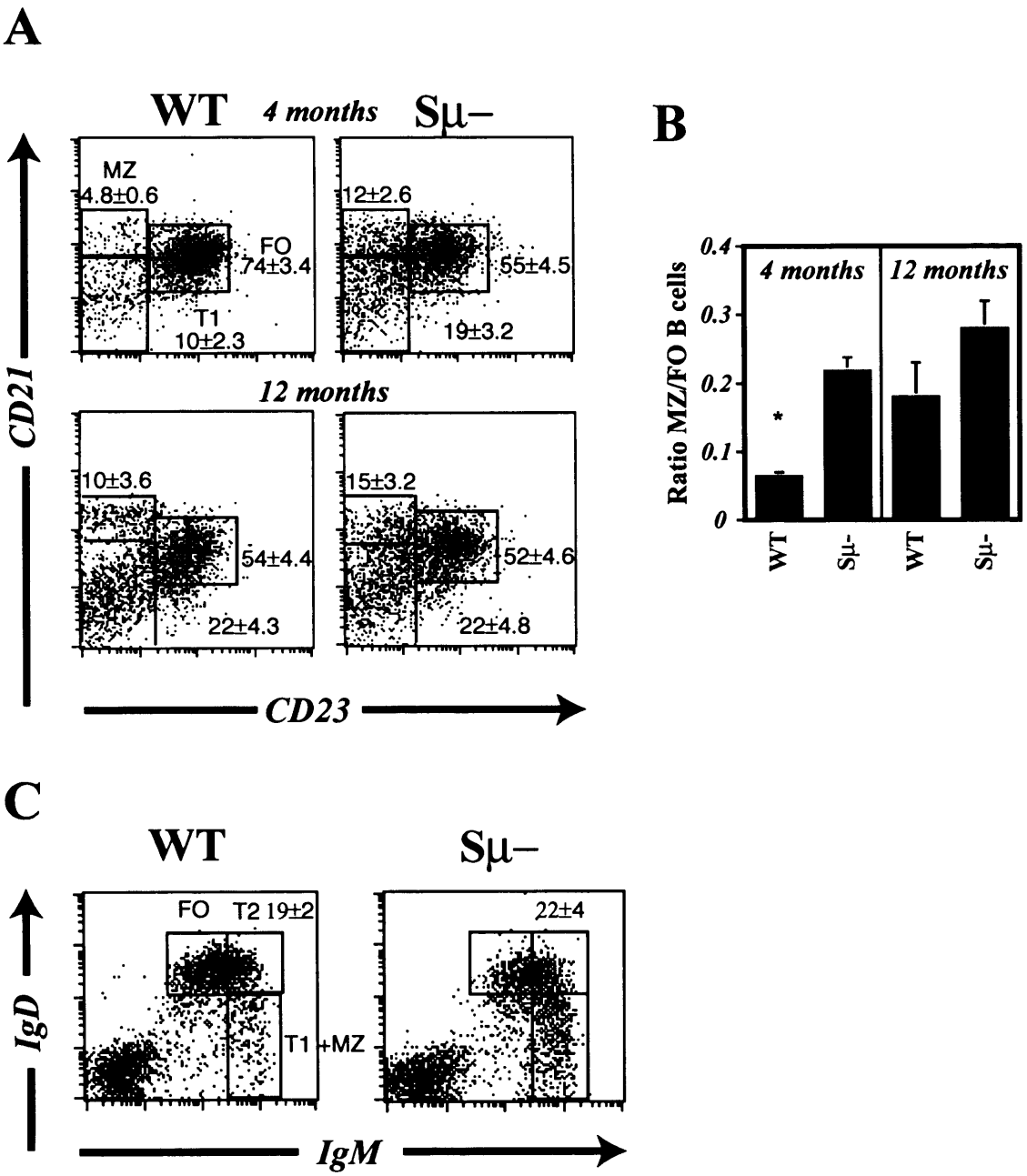

Figure 7. Splenic B cell subsets in Sμ- mice. (A) FACS analysis of spleen cells derived from Sμ- and litter-matched control (4 and 12 months), stained with anti-B220, anti-CD21, and anti-CD23. The profiles are representative of results obtained from 6–10 mice and are gated on B220+ cells. (B) Histogram showing splenic MZ:FO B cell ratios calculated using absolute cell numbers in individual cell fractions. MZ and FO were defined from FACS analysis as illustrated in (A). (C) FACS plot showing T2 B cells, stained with anti-IgM and anti-IgD. * $p<0.0002$ in Student's T Test.

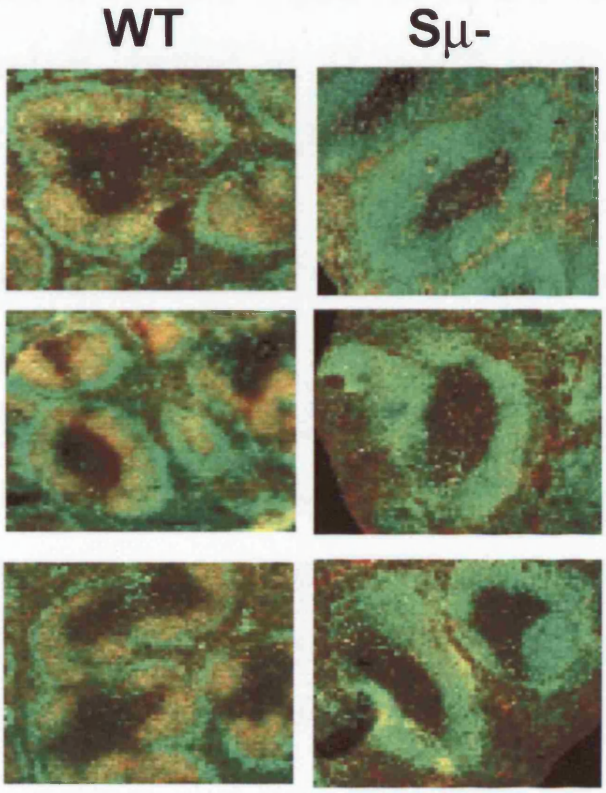

Figure 8. Histology of Sμ- mice spleens. The profiles are representative of results obtained from 6-10 mice 4 month old. Representative spleen sections derived from Sμ- and litter matched control mouse stained with anti-IgM (green) and anti-IgD (red).

Transitional type 1 B cells are expanded in Sμ- mice

Immature newly formed B cells (IgM^{high} IgD^{low} $CD21^{low}$ $CD23^{neg}$), also known as T1 or fraction E (Loder et al, 1999; Hardy and Hayakawa, 2001) were expanded in Sμ- mice (Figure 7A, 7C and Table 6), whereas the transitional subset identified as T2 (IgM^{high} IgD^{high} $CD21^{high}$ $CD23^{pos}$), remained unchanged in Sμ- mice (Figure 7C and Table 6).

Polyclonal, but not monoclonal IgM or Vμ1s transgene reverse the MZ B cell expansion

To determine whether polyclonal IgM is critical in governing the B cell compartments, two approaches were used. The first was to generate a transgenic mouse line (Vμ1s, IgH *a* allotype) in which B lymphocytes secreted a μ heavy chain, but the construct was engineered so that no transgene expression was detectable on the B cell surface (Figure 9A). These Vμ1s transgenic mice were backcrossed onto C57BL/6 mice and subsequently onto Sμ- mice to test whether MZ B cell expansion would be reversed. Consistent with the removal of the CμM exons, B lymphocyte membrane expression of the transgene was undetectable by FACS (Figure 9B), but IgM (derived only from the transgene) was present in the serum of Sμ- Vμ1s mice (Figure 10). These mice would therefore have a secreted monoclonal repertoire generated by a number of light chains pairing with the single transgenic heavy chain.

Table 6. Percentage of subpopulations of B cells in the spleen.

Naive	WT	WT Vμ1s	WT mono	WT poly	Sμ$^-$	Sμ$^-$ Vμ1s	Sμ$^-$ mono	Sμ$^-$ poly
T1	10±2	9±2	8±3	12±4	19±4	19±3	18±2	14±3
T2	19±2	21±2	16±2	14±2	22±4	22±2	22±3	17±2
MZ	4±1	4±2	4±2	3±1	12±2	12±2	12±2	3±1
Mature	67±6	66±4	71±5	70±5	47±6	47±5	48±4	64±4

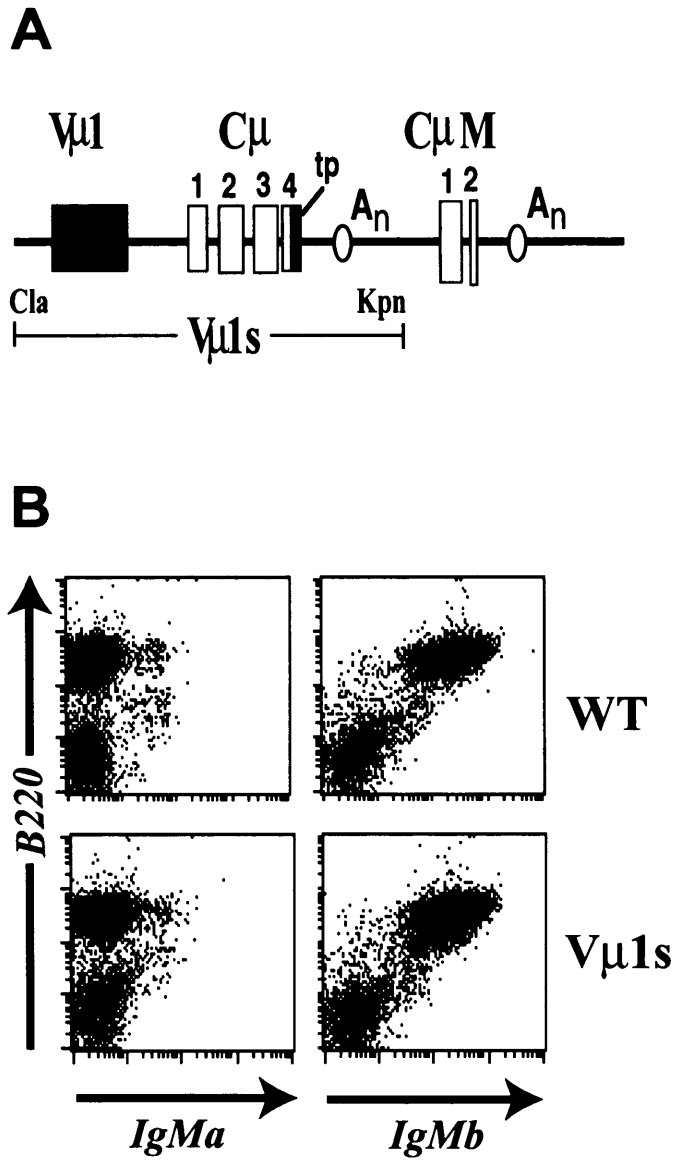

Figure 9. Transgene construct of Vμ1s. (A) The secretory μ tailpiece (tp) and the μs polyadenylation site are located within the Vμ1s construct; the μ constant region (CμM) and the μm polyadenylation have been removed from the original Vμ1 construct and are shown for information only. (B) FACS analysis of spleen cells taken from mice expressing the Vμ1s transgene using IgM allotype-specific markers. The IgM allotype of the Vμ1s transgene and the targeted Sμ- allele are *a*, whereas the C57BL/6 allotype is *b*.

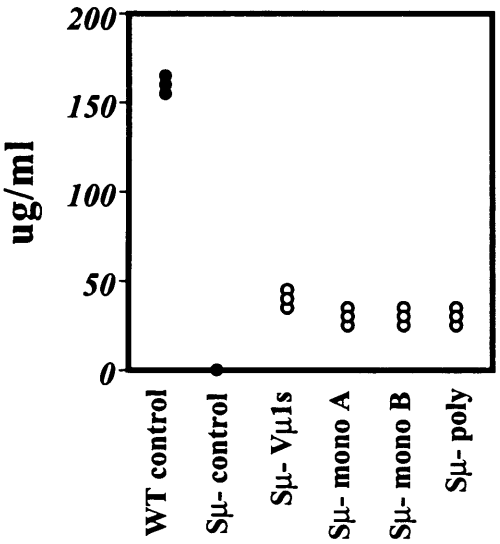

Figure 10. IgM serum concentration. Titers of serum IgM in Sμ- Vμ1s mice and in mice that had received a polyclonal (poly) preparation or two monoclonal (mono A, mono B) preparations of purified IgM measured 2 days after the final injection of the different IgM preparations (three mice per group). Samples taken 1 or 2 days after each injection were not significantly different from results shown (data not shown). Full circle IgM*b* allotype; Empty circle IgM*a* allotype.

The second method of replacing IgM was to administer a polyclonal preparation of IgM or two IgM monoclonals (mono A and mono B) i.p. every 3 days for 2 weeks. The serum concentration of these preparations during the 2 week period was identical (Figure 10). However, the levels of serum IgM obtained following the administration of IgM were only 10% of normal and fluctuated relative to the day of injection. In the Sμ- Vμ1s mice serum IgM was slightly above that found with the injected preparations although the secretion was continuous rather than intermittent as dictated by the time of injections (Figure 10).

The main conclusion from these series of IgM replacement experiments is that only the polyclonal IgM preparation returned the increased splenic MZ:FO B cell ratio found in Sμ- mice back to that observed in control mice as shown by FACS (Figure 11A and 11B) and tissue staining (Figure 10). The increased IgM staining on the periphery of the follicle seen in Sμ- mice was restored to control levels by the administration of polyclonal IgM but not by the other monoclonals or in Vμ1 Sμ- mice (Figure 12).

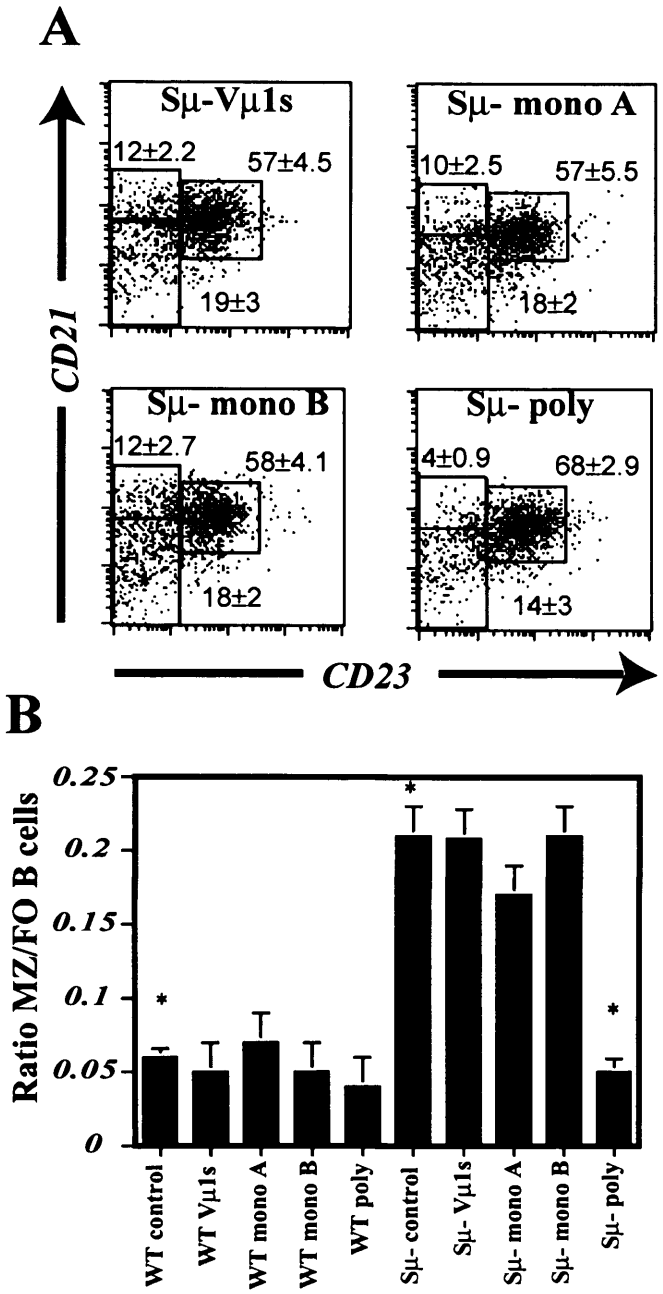

Figure 11. Polyclonal, but not monoclonal IgM or Vμ1s transgene reverse the MZ B cell expansion. (A) Representative density plots to define proportion of MZ and FO cells using CD21 and CD23 as markers after administration of monoclonal A, monoclonal B, and polyclonal IgM (poly) and in Sμ-Vμ1s mice. The density plots shown are gated on B220+ B cells. (B) Histogram showing splenic MZ:FO B cell ratio calculated using absolute cell numbers in individual cell fractions as shown in (A). MZ and FO were defined from FACS analysis, each bar represents the mean of six mice ± SD. * $p<0.0002$ in Student's T Test.

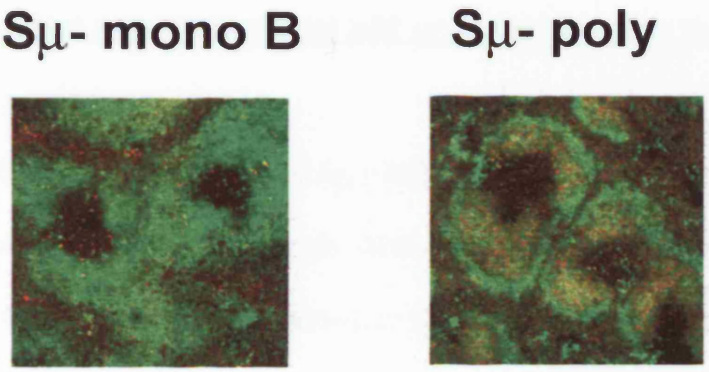

Figure 12. Histology of treated mice. Representative spleen sections derived from Sμ- mice treated with monoclonal B or polyclonal IgM stained with anti-IgM (FITC, green) and anti-IgD (Texas Red, red). All mice were between 3 and 4 month old.

Polyclonal, but not monoclonal IgM or Vμ1s transgene partially reverse the T1 B cell expansion

Figure 11A demonstrates that after two weeks of treatment with the polyclonal or monoclonal IgM preparation, the expanded newly formed (T1) B cell population found in Sμ- mice was only partially reversed by polyclonal IgM whereas the monoclonal preparation or Vμ1s transgene had no effect in this subset of immature B cells (Table 6).

Polyclonal, but not monoclonal IgM or Vμ1s reverses the peritoneal B1 B cell expansion

B1 B cells were identified by FACS using anti-IgM, ant-B220 and anti-CD5. Consistent with previous observations (Ehrenstein et. al, 1998), peritoneal B1a cells were increased in Sμ- mice. The expansion in B1 cells were reversed by polyclonal IgM but not in Sμ- mice expressing the Vμ1s transgene or the mice that had received the monoclonal IgM preparation (Figure 13A). The ratio CD5+:CD5- was calculated using absolute numbers of cells in each population (Figure 13B). Thus in this system B1 cells behaved in the same way as the marginal zone B cells.

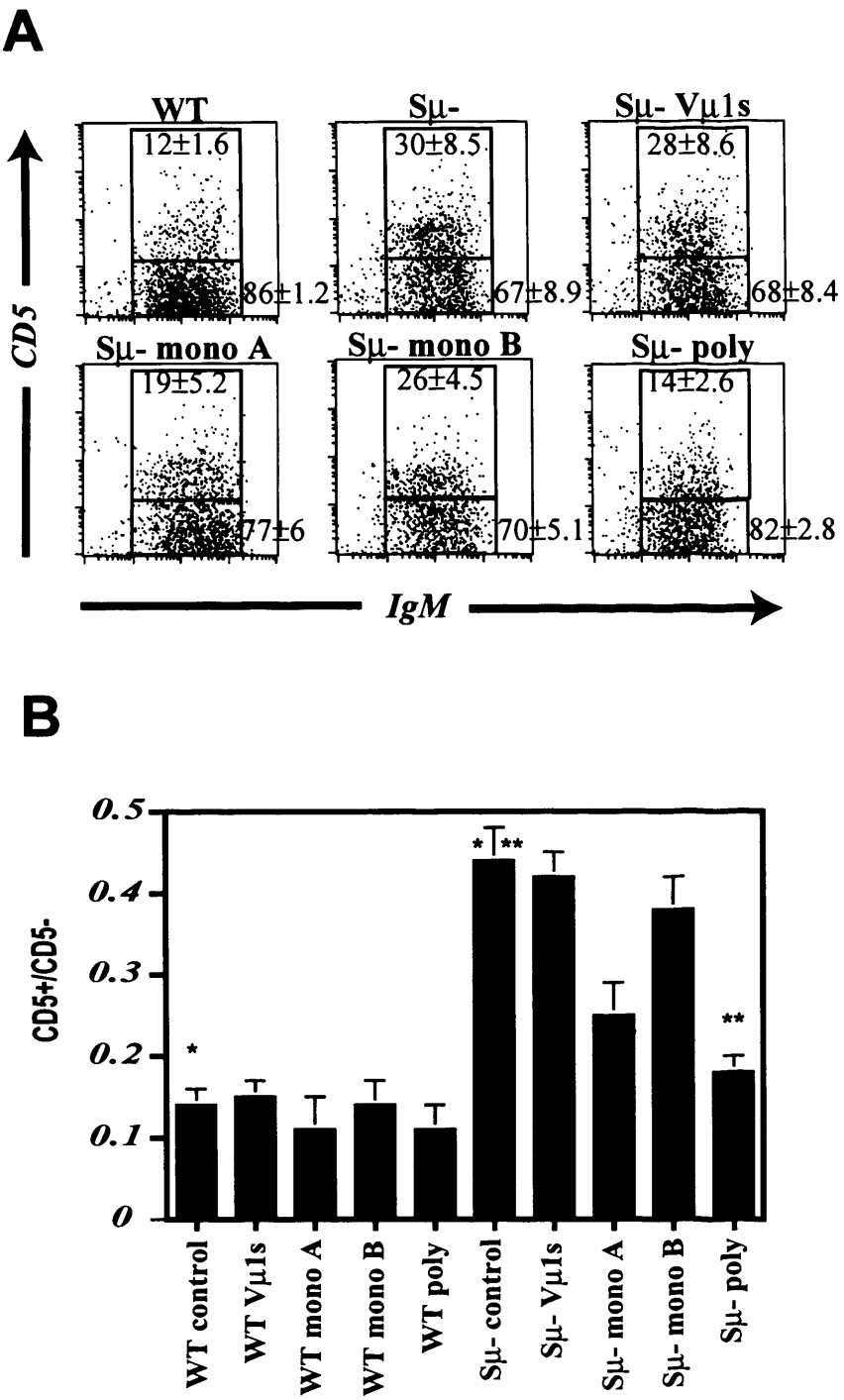

Figure 13. B1 peritoneal B cells. (A) FACS analysis of peritoneal cells gated on B220+ lymphocytes. (B) Histogram of CD5+ peritoneal B cells in naive mice. Numbers refer to the mean percentage of total B cells in each gate. The CD5+:CD5- ratio was calculated using absolute cell numbers from peritoneal washes in each group. Mono, monoclonal; poly, polyclonal. * $p<0.0007$ and ** $p<0.0001$ in Student's T Test.

Polyclonal IgM is polyreactive

To determine whether the reactivity of the different IgM preparations was associated with their ability to influence MZ and B1 cells, the binding to four different antigens (Ags) was assayed. Analysis of the binding of these IgM preparations to the Ags tested confirmed the multireactivity of polyclonal IgM which bound all four Ags strongly, monoclonal A bound two Ags strongly, whereas monoclonal B bound only one Ag (Figure 14). Thus, the Ag reactivity of the IgM preparations correlated with their ability to reverse the changes in B cell subsets found in Sμ- mice.

Sμ- follicular B cells have a higher rate of turnover than WT

In order to understand why the follicular B cell subset is reduced in Sμ- mice compared to WT and the marginal zone B cells are expanded in Sμ- mice compared to WT, cell cycling and apoptosis were measured. These techniques help to explain the homeostasis of B cells in a compartment that lacks serum IgM, thus showing its importance in the maintenance and selection of B cells in the periphery.

In vivo 5-bromo-2'-deoxyuridine (BrdU) incorporation was used to access the turnover rate of B lymphocytes in the spleen of 4 month old Sμ- mice and controls. Splenocytes were gated on B220 for B cells and then subdivided into follicular, marginal zone, transitional type 2, transitional type 1 B cells using anti-CD23, anti-CD21, anti-IgM and anti-IgD and all groups

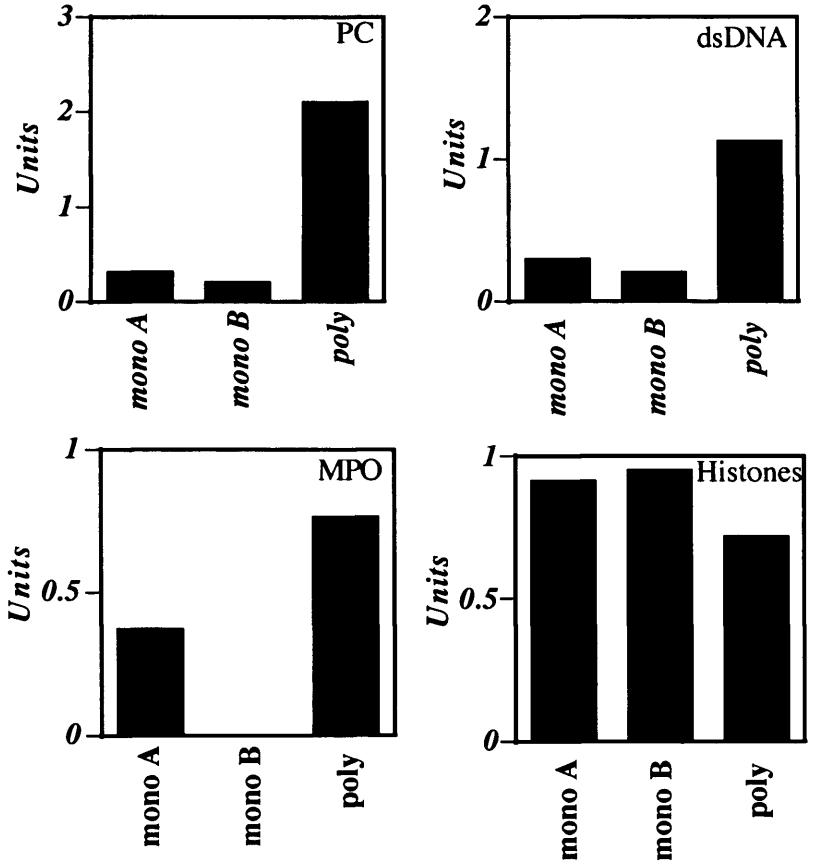

Figure 14. Reactivity of the three IgM preparations to PC, dsDNA, myeloperoxidase (MPO), and histones. Units were calculated using a serum from a 7 month old female NZB/W mouse as a reference, except for PC in which the positive control was a serum derived from a mouse immunized with PC. Mono, monoclonal; poly, polyclonal.

were stained for anti-BrdU. BrdU was administrated orally every 12 hours for 7, 14 days or administrated for 3 days following by a 14 day interval before harvesting the cells (pulse chase).

Figure 15 demonstrates that the follicular B cells in the Sμ- mice have a higher rate of turnover of cells than the control mice when these cells have been exposed to BrdU for 14 days. Marginal zone, T2, T1 and B1 B cells from Sμ- mice have similar turnover rates of cells from the WT mice. These results suggest that follicular B cells, but not the other B cell populations are sensitive to the loss of serum IgM.

Sμ- peritoneal B1 B cells have a lower rate of turnover than WT

In vivo BrdU incorporation was used to access the turn over rate of B lymphocytes in the peritoneum cavity of 4 month old Sμ- mice and controls. Cells from the peritoneal cavity were gated on B220 for B cells and then subdivided in 2 different groups. CD5+IgM+ for B1 B cells and IgM+CD5- for B2 B cells and all groups were stained for anti-BrdU.

Figure 16 shows that a reduction of BrdU incorporation was seen in B1 B cell after 14 days of continuous administration of BrdU in the Sμ- mice. These mice have a higher number of B1 B cells in the peritoneal cavity when compared to WT, thus these results showed that these cells are not cycling as much, and probably remain in the peritoneal cavity longer. A strong positive signal via IgM is probably needed for B1 B cells to enter and

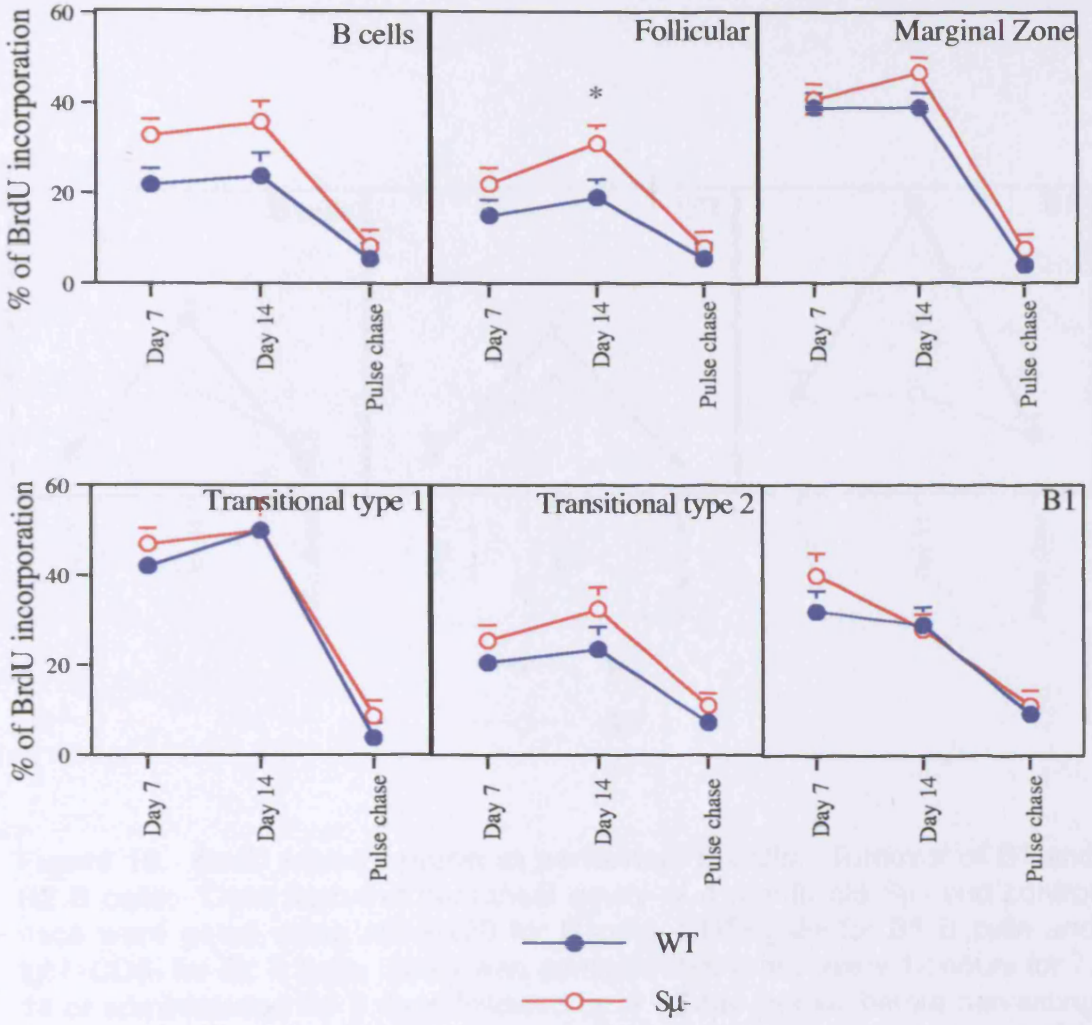

Figure 15. BrdU incorporation in splenic B cells. Turnover of B cells, follicular, marginal zone, tansitional type 2, transitional type 1 and B1 B cells. Splenocytes of 4 month old Sμ- and control mice were stained with anti-CD21, anti-CD23, anti-IgM, anti-IgD, anti-CD5 and anti-BrdU. B cells were gated using anti-B220. BrdU was administrated orally every 12 hours for 7, 14 or administrated for 3 days followed by a 14 day interval before harvesting the cells (pulse chase). Each point represents the mean of 4 mice ± SD. *p<0.001 in Student's T Test.

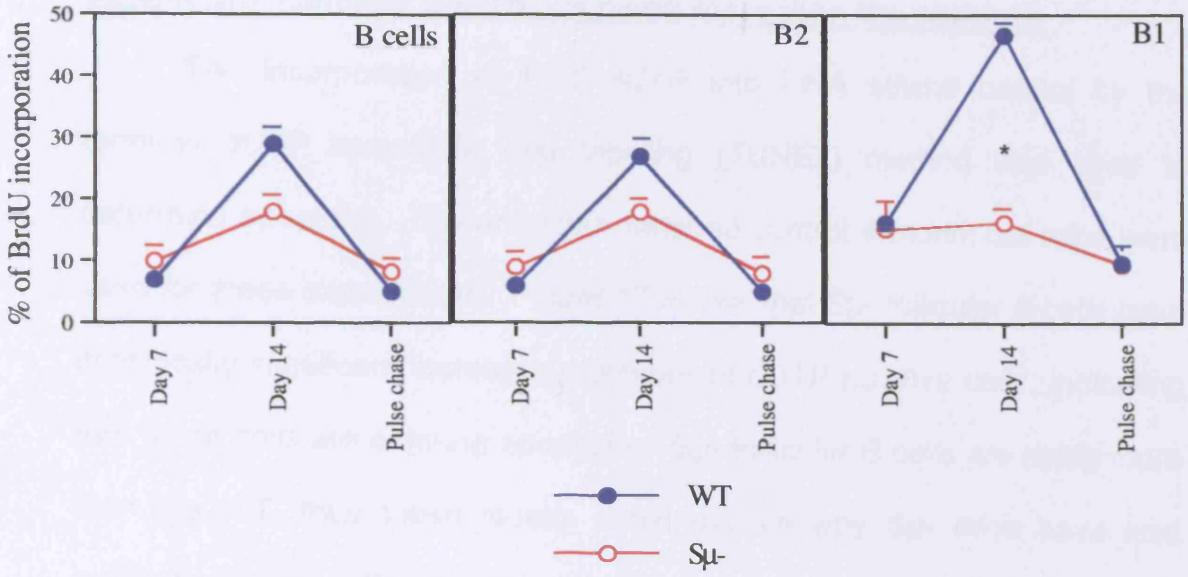

Figure 16. BrdU incorporation in peritoneal B cells. Turnover of B1 and B2 B cells. Cells from the peritoneal cavity of 4 month old Sμ- and control mice were gated using anti-B220 for B cells, CD5+IgM+ for B1 B cells and IgM+CD5- for B2 B cells. BrdU was administrated orally every 12 hours for 7, 14 or administrated for 3 days followed by a 14 day interval before harvesting the cells (pulse chase). Each point represents the mean of 4 mice ± SD. *$p<0.0001$ in Student's T Test.

maintain proliferation, implying that natural IgM has different effects in different compartments as well as different populations of B cells.

Splenic Sμ- follicular B cells are dying more than the controls

The incorporation of FITC-dUTP into DNA strand breaks by the terminal dUTP nucleotide end labeling (TUNEL) method was used to determine apoptosis. Sμ- and litter matched control 4-month-old mice were used for these experiments. Figure 17 shows that Sμ- follicular B cells have statistically significant increased numbers of dUTP positive cells, indicating that these cells are entering apoptosis. Sμ- follicular B cells are dying more than the WT, thus these results could explain why Sμ- mice have less number of follicular B cells. There were no differences in the number of cells entering apoptosis in the other B cell subpopulations (MZ, T1, T2 and B1 B cells).

Sμ- peritoneal B1 B cells are dying less

The incorporation of FITC-dUTP into DNA strand breaks by the terminal dUTP nucleotide end labeling (TUNEL) method was used to determine apoptosis. Figure18 shows that peritoneal B1 B cells positive for IgM, CD5 and B220 have a decreased number of dUTP positive cells, indicating that these cells are entering apoptosis, but at a smaller extent than WT B1 B cells. The only statistically significant difference between the control and Sμ- mice was found in the B1 B cell pool (Figure 18).

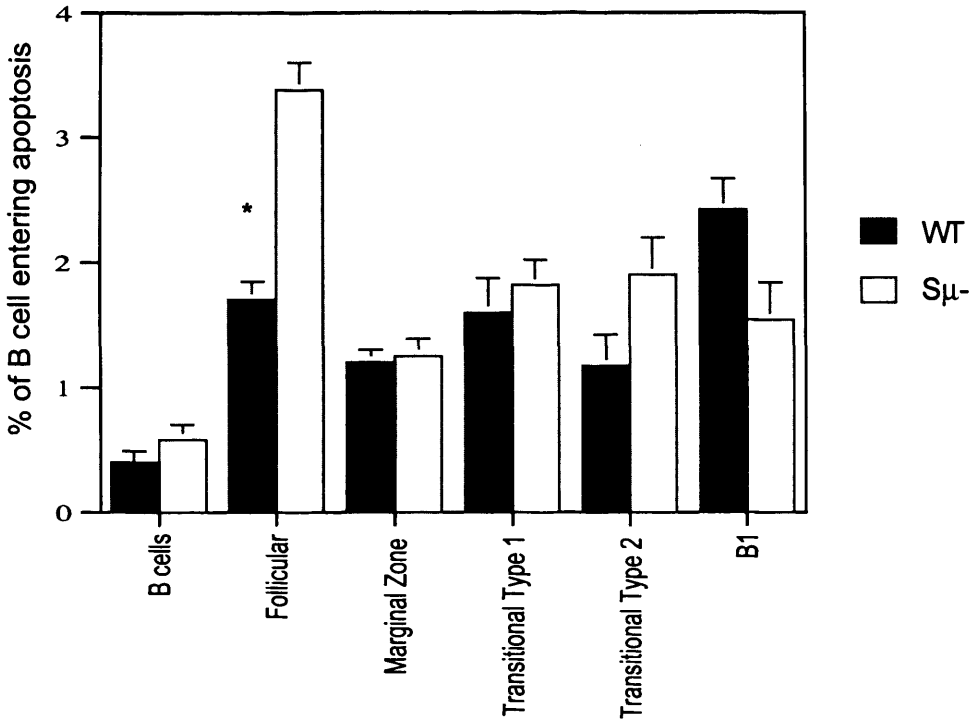

Figure 17. Apoptosis in splenic B cells. 4-month-old Sμ- and litter matched controls were labeled for the incorporation of FITC-dUTP into DNA strand breaks by the terminal dUTP nucleotide end labeling (TUNEL) by FACS. B cells, follicular, marginal zone, transitional type 1, transitional type 2 and B1 B cells were stained with anti-CD21, anti-CD23, anti-IgM, anti-IgD, anti-CD5 and anti-dUTP. B lymphocytes were gated on B220. Each bar represents the mean of 4 mice ± SD. *$p<0.0001$ in Student's T Test.

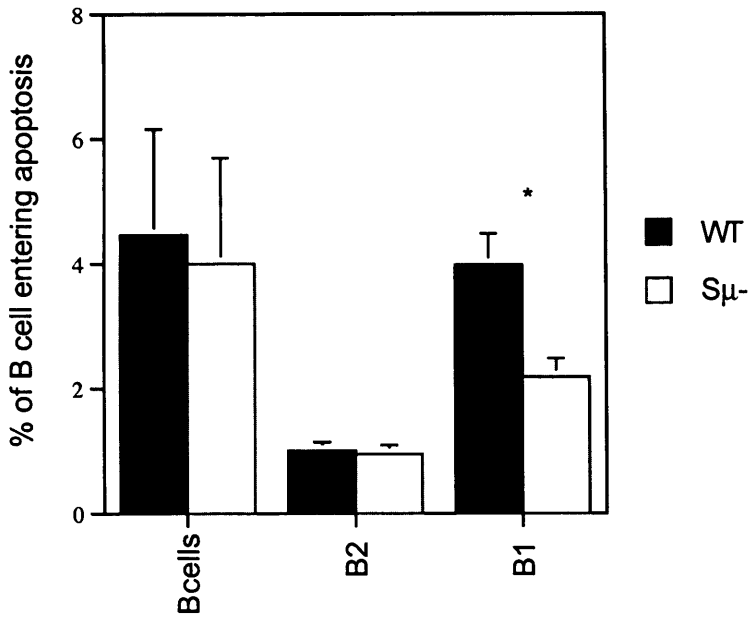

Figure 18. Apoptosis in peritoneal B cells. 4-month-old Sμ- and litter matched controls were labeled for the incorporation of FITC-dUTP into DNA strand breaks by the terminal dUTP nucleotide end labeling (TUNEL) by FACS. Cells from the peritoneal cavity were gated on B220 for B cells and then subdivided in 2 different groups. CD5+IgM+ for B1 B cells and IgM+CD5- for B2 B cells. Each bar represents the mean of 4 mice ± SD. *$p<0.004$ in Student's T Test.

B1 B cells are behaving in a different way compared to B2 cells, they cycle and die less in the absence of serum IgM, whereas B2 cells in the spleen have a higher rate of cell turnover and apoptosis.

Calcium mobilisation following IgM cross-linking shows no differences between WT and Sμ- mice

CD21, CD23, CD5 and B220 markers were used to identify the B cell subtypes, as described above. Calcium flux was measured by calculating the ratio of calcium bound indo-1 (FL5) and unbound (FL4) versus time. Figure 19 shows that B cells when stimulated with agonist anti-IgM give rise to calcium responses. Splenocytes of 4 month old wild type and Sμ- mice have the same pattern of calcium response when stimulated with different concentrations of anti-IgM ranging from 0.5ug/ml to 30ug/ml, figure 19 showing results with 10ug/ml of anti-IgM. Subsets of B cells were analysed, MZ B cells have an increased response, whereas FO, T1, T2 and B1 B cells have about the same amount of calcium mobilisation.

Cells from the peritoneal cavity of 4 month old WT and Sμ- mice stained for anti-B220 and anti-CD5 were also analysed and there was no difference in the amount of calcium mobilisation between the WT and Sμ- mice when B1 and B2 B cells were compared (Figure 20). However, there were differences between B1 B cells resident in the peritoneum which showed hyporesponsiveness (no BCR mediated calcium response) and elevated basal calcium flux comparable to B2 B cells from the peritoneum or

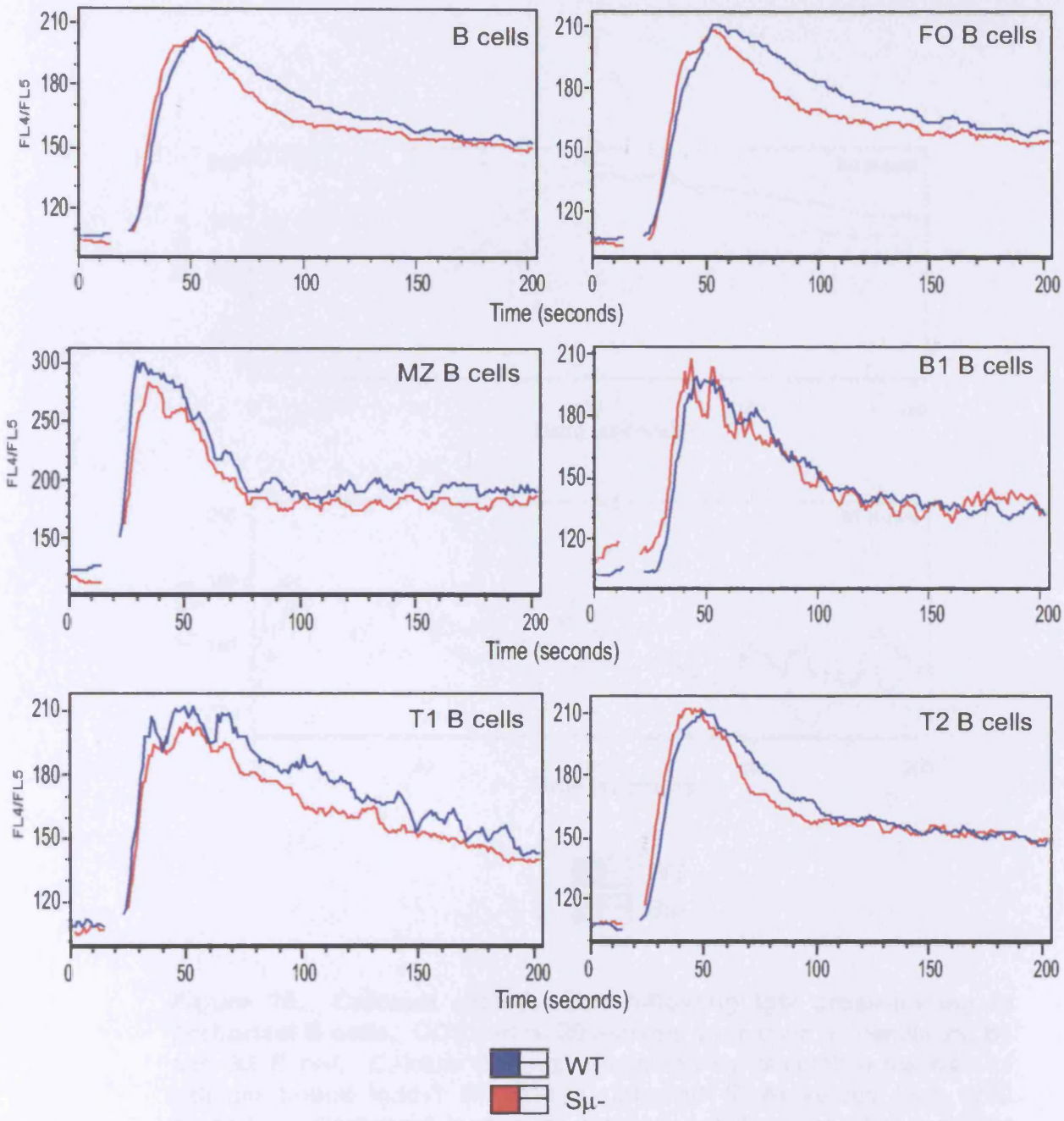

Figure 19. Calcium mobilisation following IgM cross-linking in splenic B cells. CD21, CD23, CD5 and B220 markers were used to identify the B cell subtypes, as described before. Calcium flux was measured by calculating the ratio of calcium bound indo-1 (FL5) and unbound (FL4) versus time (200 seconds). Splenocytes of 4 month old Sμ- and litter matched control were stimulated with the agonist anti-IgM (fab2), at a concentration of 10ug/ml, few seconds before being analysed by FACS. The experiment was repeated 4 times giving consistent results.

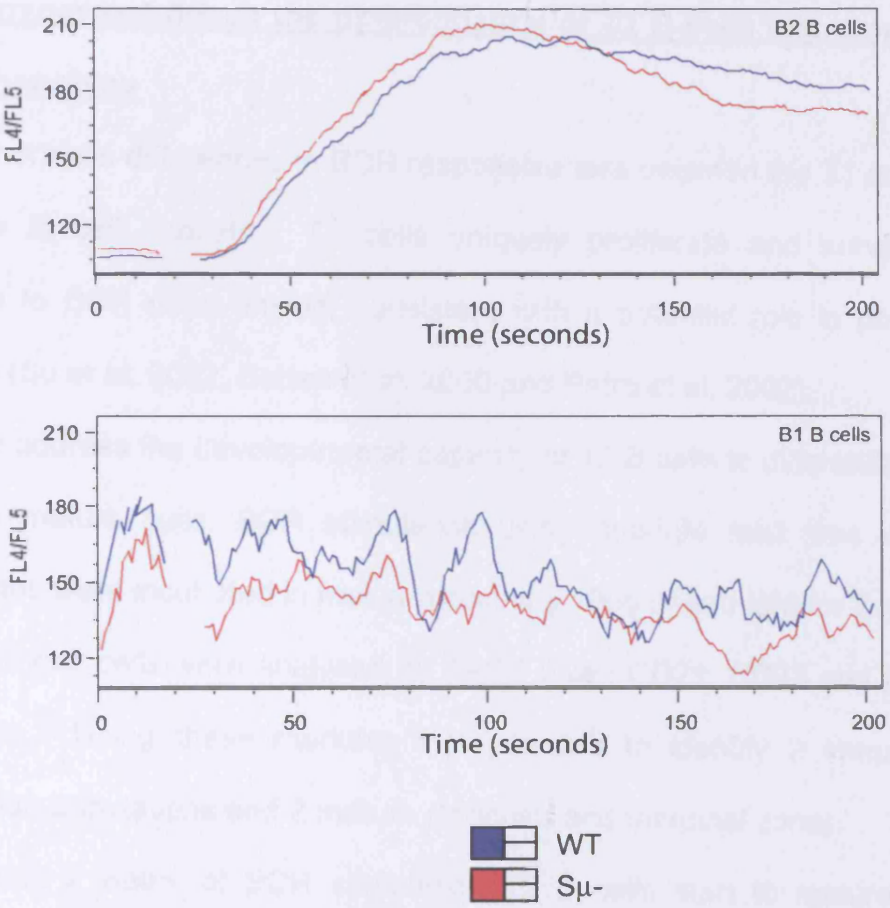

Figure 20. Calcium mobilisation following IgM cross-linking in peritoneal B cells. CD5 and B220 markers were used to identify the B1 and B2 B cell. Calcium flux was measured by calculating the ratio of calcium bound indo-1 (FL5) and unbound (FL4) versus time (200 seconds). Peritoneal lavage of 4 month old Sμ- and litter matched control were stimulated with the agonist anti-IgM (fab2), at a concentration of 10ug/ml, few seconds before being analysed by FACS. The experiment was repeated 4 times giving consistent results.

B1 B cells from the spleen. These results are representative of 4 experiments.

BCR engagement drives the differentiation of T2 B cells into a mature B cell phenotype

There are differences in BCR responsiveness between the T1 and T2 immature B cell subsets. T2 cells uniquely proliferate and survive in response to BCR cross-linking, consistent with a potential role in positive selection (Su et al, 2002, Batten et al, 2000 and Petro et al, 2002).

To address the developmental capacity of T2 B cells to differentiate in vitro into mature cells, BCR stimulation using anti-IgM fab2 was used. Splenocytes were incubated in media containing 10ug of anti-IgM for 2 hours and then these cells were analysed by FACS using CD21, CD23 and B220 for B cells. Using these markers it is possible to identify 2 immature (transitional) populations and 2 mature (follicular and marginal zone).

Within 2 hours of BCR stimulation T2 B cells start to mature, as judged by the down regulation of CD21. Figure 21 shows the ratio of T2:FO calculated using the absolute number of cells, the number of cells decreased within 2 hours of BCR stimulation in the control and Sμ- mice. No changes were found in the other B cell subsets. These results show that the WT as well as the Sμ- T2 B cells are capable to mature in vitro upon BCR stimulation when identified using CD23, CD21 and B 220 as cell markers.

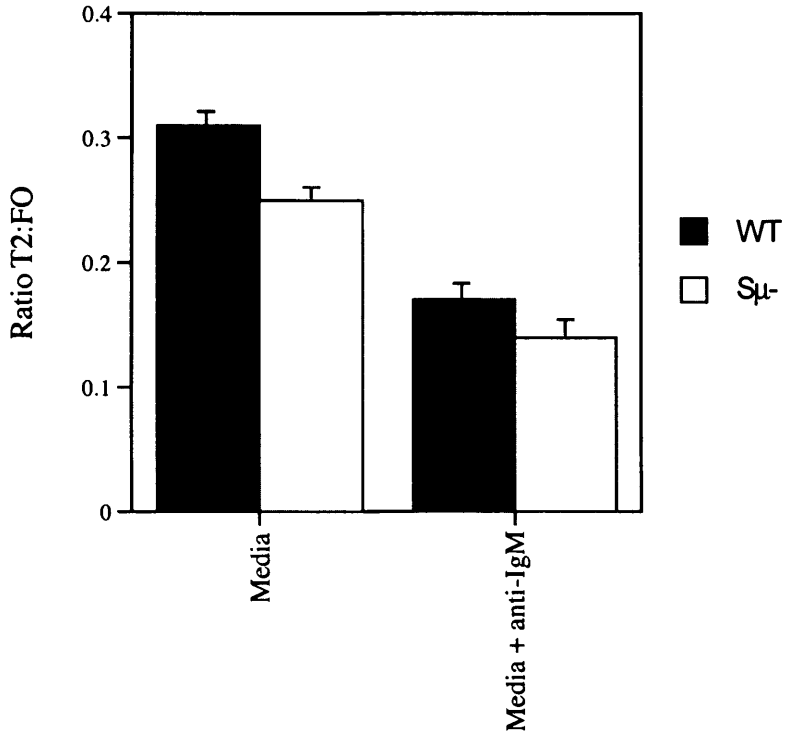

Figure 21. BCR engagement drives the differentiation of T2 B cells into a mature B cell phenotype. Splenocytes of 4 month old Sμ- and litter matched control were incubated in media containing 10ug of anti-IgM (fab2) or media alone for 2 hours. Follicular and Transitional Type 2 B cells were stained with anti-CD21, anti-CD23 and anti-B220. The ratio of T2:FO was calculated using absolute cell numbers from each fraction derived from FACS analyses. Each bar represents the mean of 4 mice ± SD.

BCR engagement drives B cells to blast normally

To confirm that B cells from Sμ- mice were responding well to BCR stimulation, splenic cells were stimulated in vitro with a range of concentrations of anti-IgM (fab2) for 24 hours. Cells were harvested and analysed by FACs looking at the forward and sitter scatter (Figure 22). B cells from Sμ- mice as well as the litter matched control were able to blast in response to BCR crosslinking.

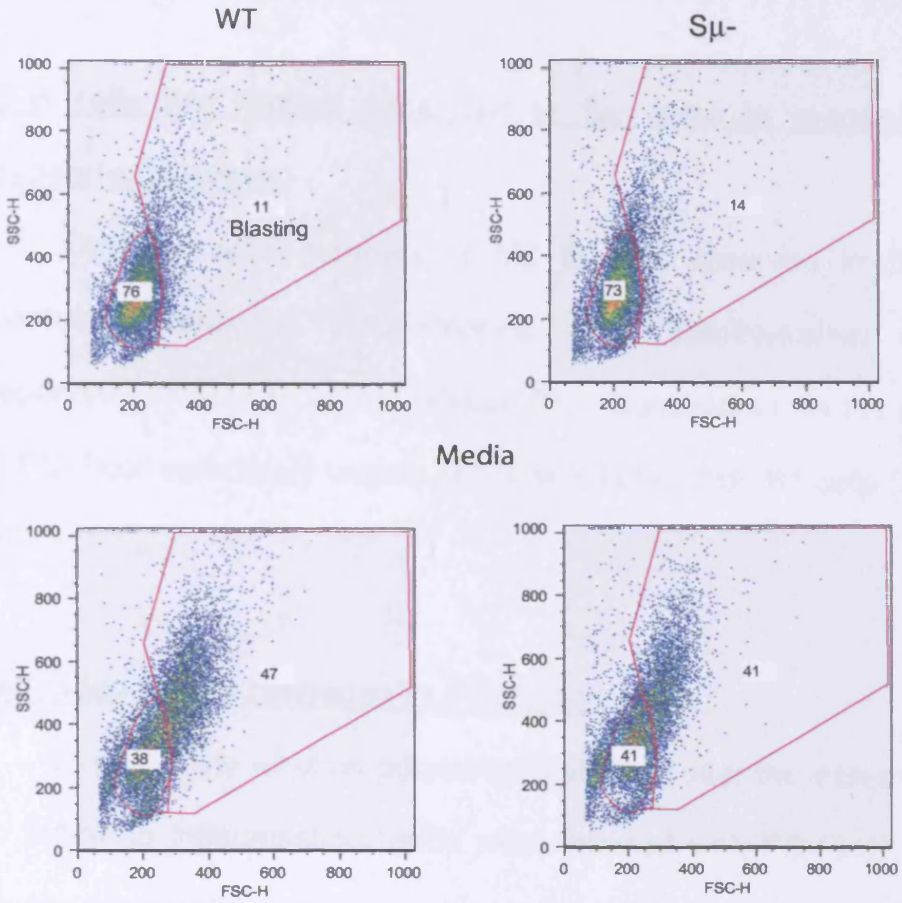

Figure 22. Blasting B cells cells upon BCR stimulation. Splenic cells were stimulated in vitro with media alone or media + anti-IgM (fab2) for 24 hours. Cells were harvested, and analysed by FACS. Two gates were made, one for blasting B cells and one for resting B cells, the numbers shown are the percentage of cells in each gate. The plots are representative of 4 experiments.

T independent response

MZ B cells are further expanded in Sμ- mice in response to a T-independent antigen

The increased numbers of MZ B cells observed in Sμ- mice is accentuated following intraperitoneal (i.p.) administration of the T-independent antigen PC-Ficoll (Figure 23). Consistent with the observation that PC-Ficoll selectively targets MZ B cells rather than B1 cells (Amlot et al, 1985).

T cell independent response to PC-Ficoll

To determine whether polyclonal IgM could alter the increased MZ:FO ratio following immunisation, mice were injected with PC-Ficoll. The day following the injection either polyclonal or monoclonal IgM was administered for two weeks using the same protocol as described above. The increased IgG_3 immune response seen in Sμ- mice was partially reversed by the administration of polyclonal IgM (Figure 24A) and these changes paralleled the alterations in the MZ/FO B cell ratio (Figure 24B).

Table 7 shows the percentage of marginal zone, T1, T2 and follicular/mature B cells following immunisation with PC-Ficoll.

Table 7. Immunised mice with PC-Ficoll. Percentage of subpopulations of B cells in the spleen.

Immunised	WT	WT Vμ1s	WT mono	WT poly	Sμ$^-$	Sμ$^-$ Vμ1s	Sμ$^-$ mono	Sμ$^-$ poly
T1	10±1	10±2	10±2	13±2	15±1	17±3	17±2	16±2
T2	21±2	28±4	22±2	18±3	27±1	29±3	20±2	18±3
MZ	7±1	8±1	4±2	4±2	16±2	15±3	13±2	9±3
Mature	61±3	58±2	63±3	68±3	41±4	38±4	49±3	57±7

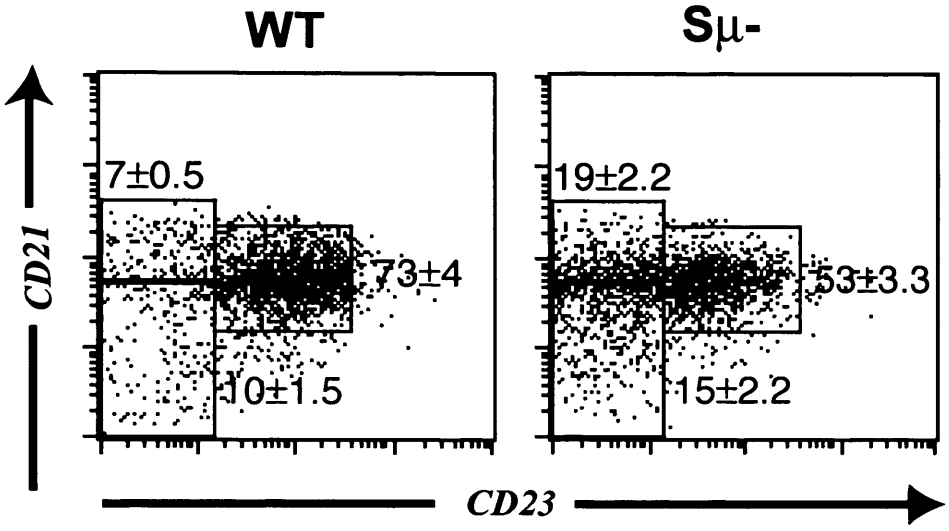

Figure 23. Splenic B cell subsets in immunised Sμ- mice. FACS analysis of spleen cells derived from Sμ- and litter-matched controls two weeks following immunisation with PC-Ficoll, stained with anti-B220, anti-CD21 and anti-CD23. The profiles are representative of results obtained from 6-10 mice 4 month old, and are gated on B220 positive cells.

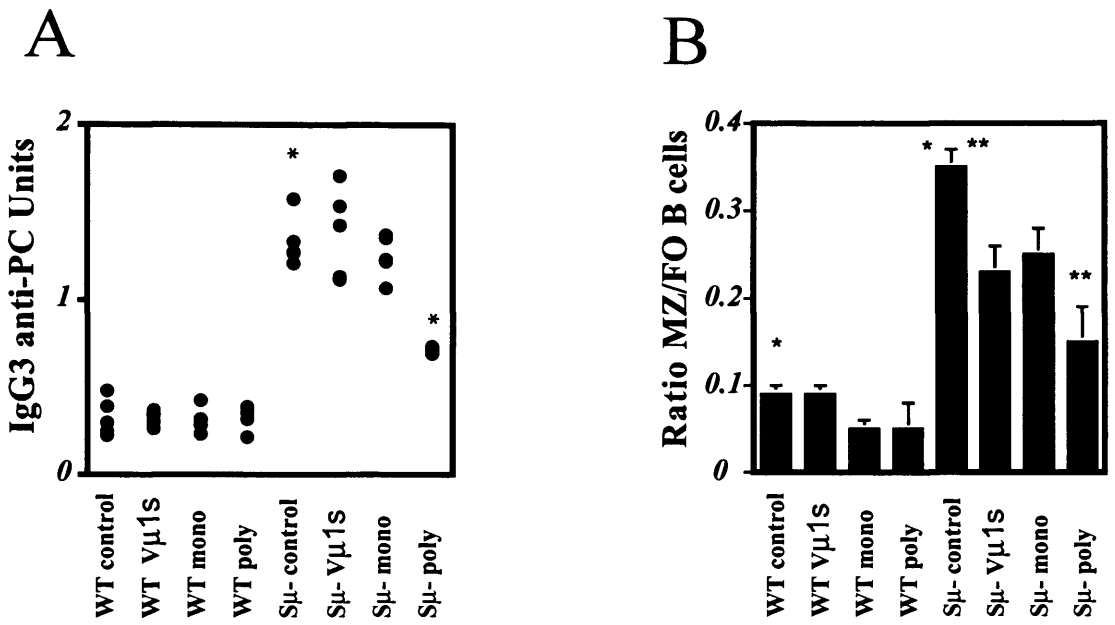

Figure 24. T cell independent response to PC-Ficoll. (A) IgG3 anti-PC reactivity 2 weeks after immunisation with 5μg PC-Ficoll (5 mice per group, * $p<0.0005$ in Student's t-test). Some mice as indicated were also treated with either polyclonal or monoclonal IgM starting 24 hours after the administration of PC-Ficoll. (B) MZ:FO ratio of splenic B cells two weeks after immunisation corresponding to the groups shown in (A). *$p<0.0003$ and **$p<0.003$ in Student's t-test.

Autoimmunity in Sμ- lpr mice

IgG anti-DNA antibodies in *lpr* and Sμ- *lpr* mice

Serum IgM deficient mice develop autoantibodies and mild glomerunephritis with age (Ehrenstein et al, 2000, and when intercrossed into MRL/*lpr,* these mice develop a more severe lupus like disease (Boes et al, 1998a). To further determine the role of IgM in autoimmunity, IgM deficient mice were intercrossed into *lpr* mutant mice in the BL/6 background, which develops autoimmunity, but not as severe as in the MRL background. Sμ-*lpr*, *lpr* and litter matched controls were followed for 12 months. There were no differences in the level of anti-DNA antibodies (Figure 25) or proteinuria (++), the only difference was that the Sμ-*lpr* mice developed a more severe lymphoproliferation. Sμ-*lpr* mice had a 4-fold ($2.9x10^8$ cells ± 1.2) and *lpr* 2-fold ($1.2x10^8$ cells ± 0.6) increase in the size of the spleen compared to WT ($7x10^7$ cells ± 3), Sμ- mice had similar number of cells compared to WT. The enlarged lymph nodes of the neck were counted. Sμ-*lpr* mice had 3 LN weighting 0.25g ± 0.10 each, 50% greater lymph node enlargement in the neck compared to *lpr* mice (2 LN, 0.15g ± 0.07 each). Mean of 5 mice per group and SD.

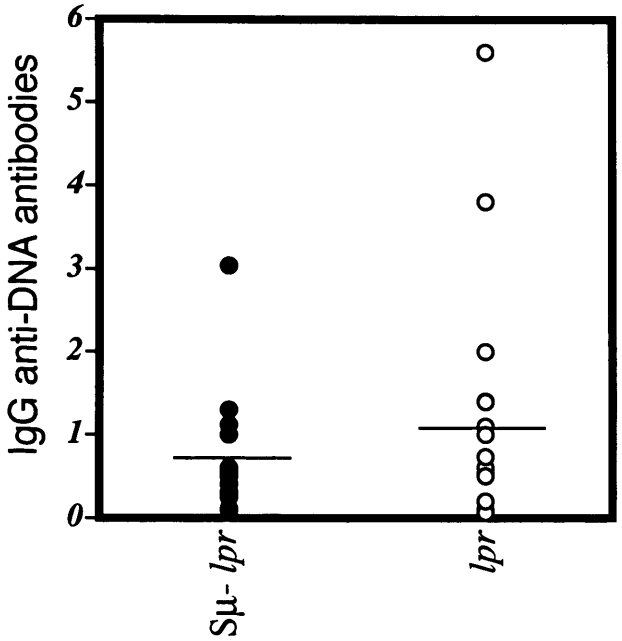

Figure 25. IgG anti-dsDNA antibodies. ELISA of 5 month old Sμ-*lpr* (solid circles) and *lpr* (open circles) mice. Blood was taken from the tail vein. Dots represent single mouse (15 per group). Means are indicated by the horizontal bar. Results given by Units (1 Unit=1.000 OD of a MRL*lpr*)

Double Negative T cells in Sμ-*lpr* and *lpr*

Lpr mice are known to a have double negative (DN) T cell population that has escaped negative selection. DN T cells bear surface markers, which are not characteristic of normal resting T cells, such as B220 (Cohen and Eisenberg, 1991). B cells also manifest cell surface abnormalities due to Fas mutation, such as early polyclonal B activation, rapid capping of immunoglobulin receptors, and high expression of class II MHC molecules (Reap et al, 1996).

FACS analyses were performed using B220, IgM and CD3 to see if the B, T and DN T splenic cells were different in Sμ-*lpr* compared to *lpr* mice. Sμ-*lpr* and *lpr* mice had less T cells and more B cells at 4 month of age when compared to 12 old month mice, Sμ-*lpr* mice had more DN T cells characterised by B220/CD3 positive cells at all ages (Figure 26). DN T cells in the lymph nodes were increased at 12 month of age (Figure 27).

B cell subsets in Sμ-*lpr* mice

In order to study the B cell population in the Sμ-*lpr* mice, FACS was performed using CD19 instead of B220 because it stains the DN T cells as well, IgM, IgD, CD21 and CD23. Cells were gated for lymphocytes and to determine the percentage of MZ, FO, T1, T2 and B1 B cells, they were gated for B cells only. Splenic and peritoneal cells of 4 and 12 month old Sμ-*lpr*, Sμ-, *lpr* and litter matched controls were used for the cell surface staining and analysed by FACS. Figure 28 and 29 show the percentage and SD of

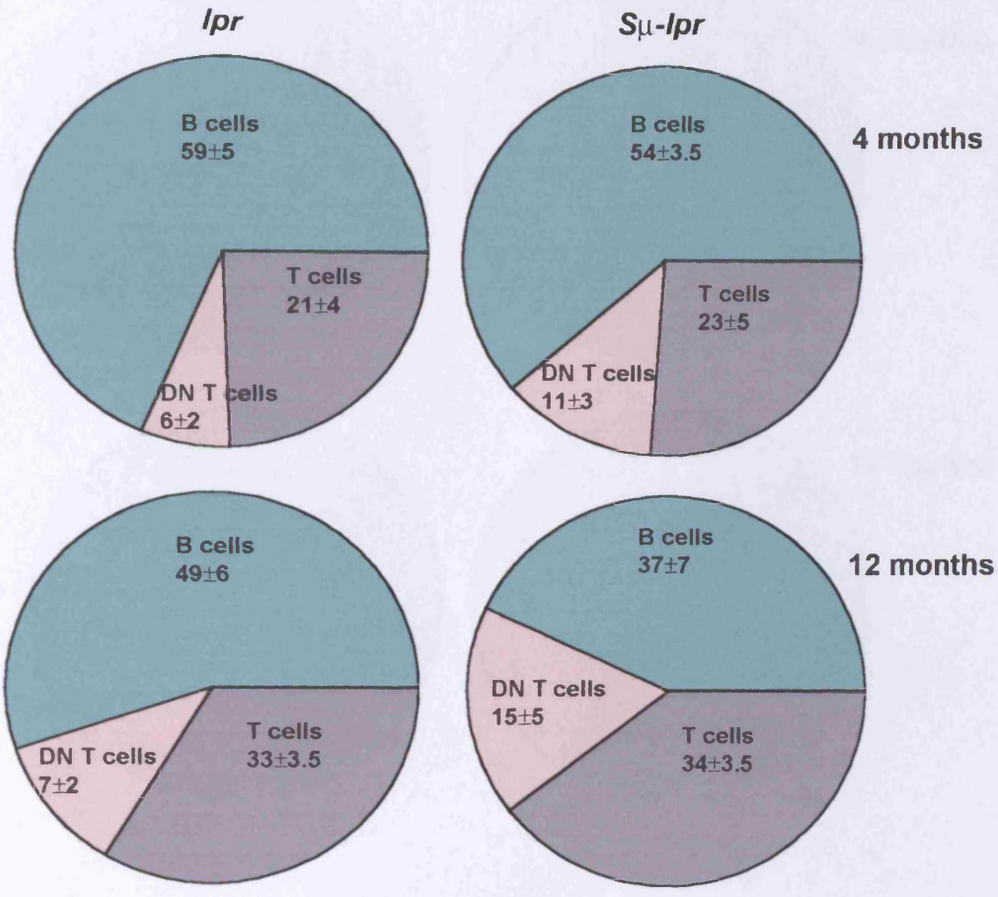

Figure 26. Splenic double negative T cells, B and T cells. Histogram showing the percentage of B, T and DN T cells in the spleen. Cells were gated for lymphocytes and stained using anti-CD3, anti-IgM and anti-B220. Sμ-*lpr and lpr* mice were 4 and 12 month old (5 mice per group ± SD).

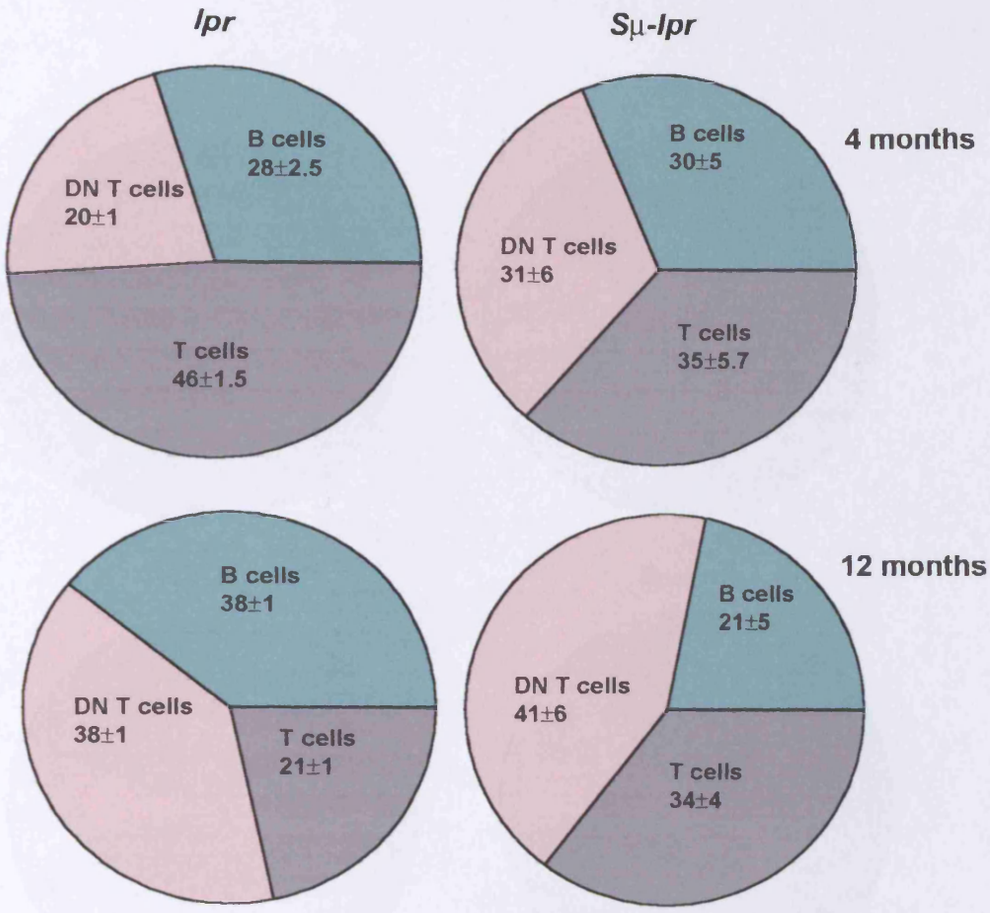

Figure 27. Lymph node double negative T cells, B and T cells. Histogram showing the percentage of B, T and DN T cells in the lymph node. Cells were gated for lymphocytes and stained using anti-CD3, anti-IgM and anti-B220. Sμ-*lpr and lpr* mice were 4 and 12 month old (5 mice per group ± SD).

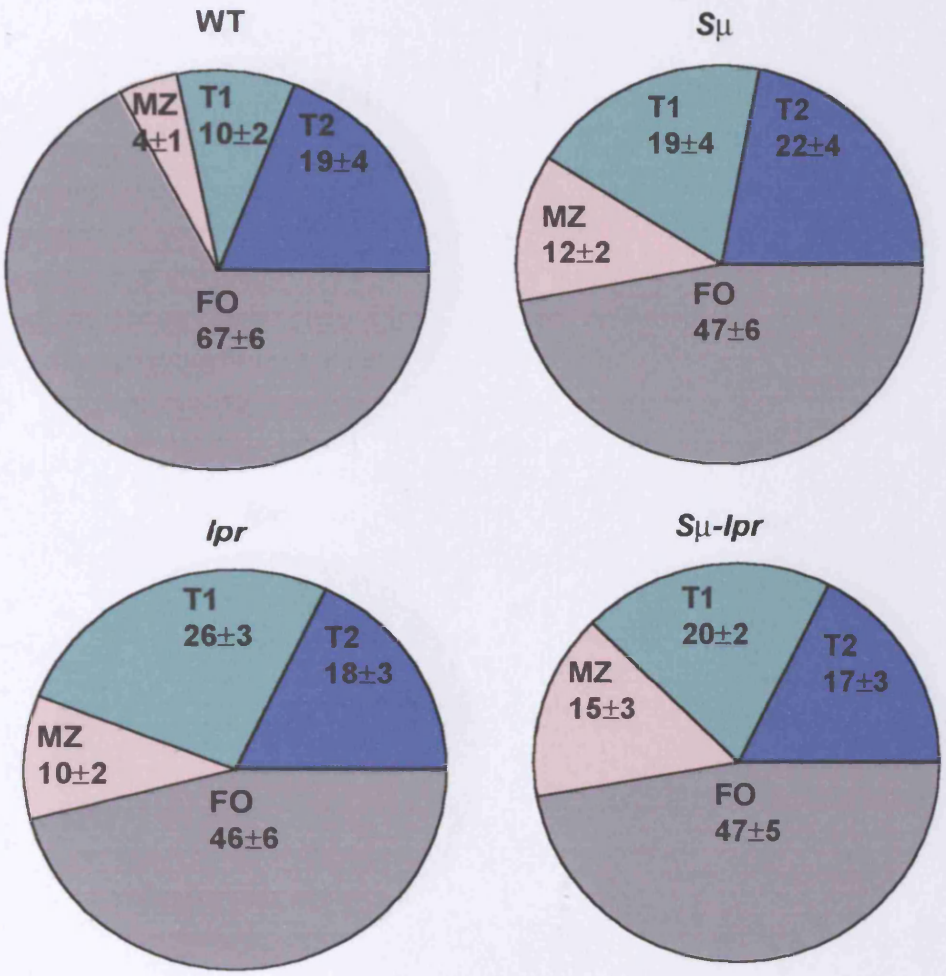

Figure 28. B cell subsets in 4 month old mice. Histogram showing the percentage of Follicular, Marginal Zone, Transitional type 1 and Transitional Type 2 B cells in the spleen. Cells were gated for lymphocytes and stained using anti-CD23, anti-CD21, anti-IgM and anti-B220. Sμ-, Sμ-*lpr, lpr* and control mice were 4 month old (5 mice per group ± SD).

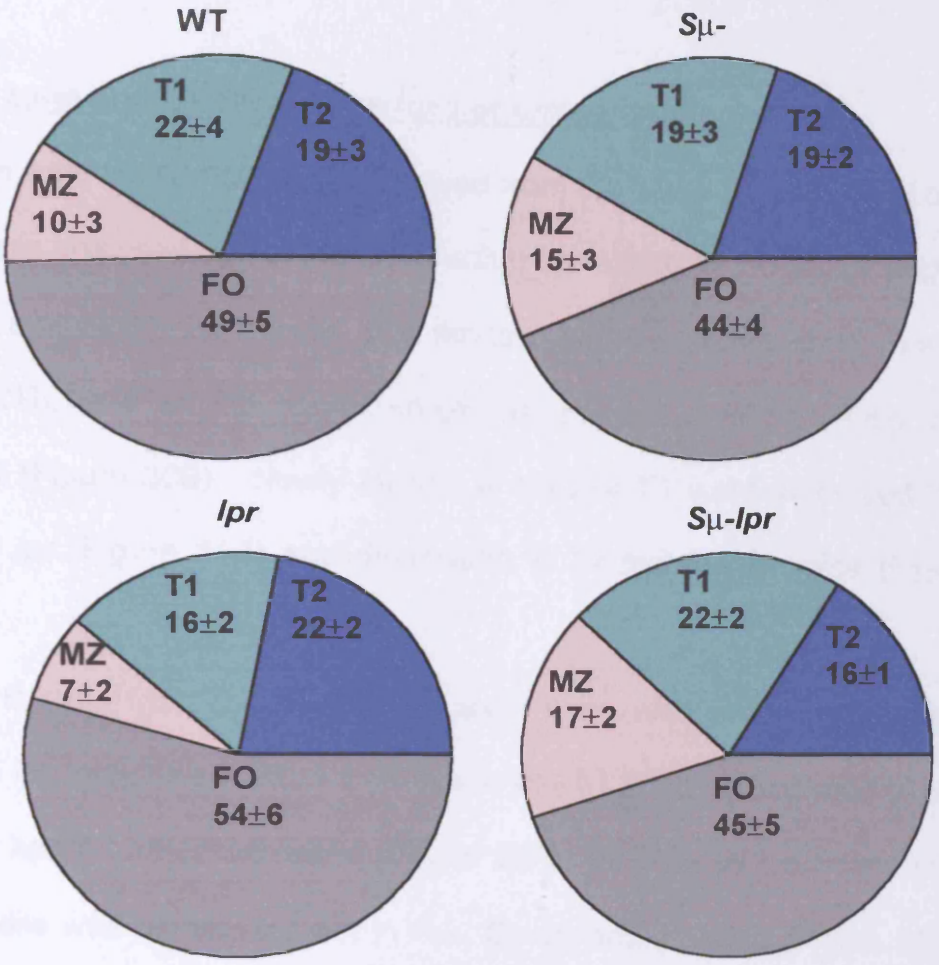

Figure 29. B cell subsets 12 month old mice. Histogram showing the percentage of Follicular, Marginal Zone, Transitional type 1 and Transitional Type 2 B cells in the spleen. Cells were gated for lymphocytes and stained using anti-CD23, anti-CD21, anti-IgM and anti-B220. Sμ-, Sμ-*lpr, lpr* and control mice were 12 month old (5 mice per group ± SD).

T1, T2, MZ and FO B cell subpopulations in the spleen of 4 and 12 month old mice.

Marginal zone and B1 B cells are further expanded Sμ-*lpr*

The ratio of MZ:FO B cells (derived from the absolute number of cells in each gate analysed by FACS) was further increased in the Sμ-*lpr* (Figure 30A), not statistically significant at 4 months, but significant at 12 months (Figure 30B), and in the 12-month-old *lpr* mice the MZ:FO ratio was decreased (Figure 30B). Newly formed B cells or T1 were increased in 4 month old *lpr* (Figure 31A) and decreased in 12 months *lpr* mice (Figure 31B).

B1 B cells from the peritoneal cavity were also analysed in these mice. The *lpr* mice had a slight increase in the B1 B cells compared to WT and Sμ-*lpr* had it further increased (Figure 32A). In older WT mice the ratio of B1/B2 cells was higher, but not in Sμ-, Sμ-*lpr* and *lpr* mice (Figure 32B). There were no evident differences in the B1 B population in the spleen of Sμ-*lpr* and *lpr* mice.

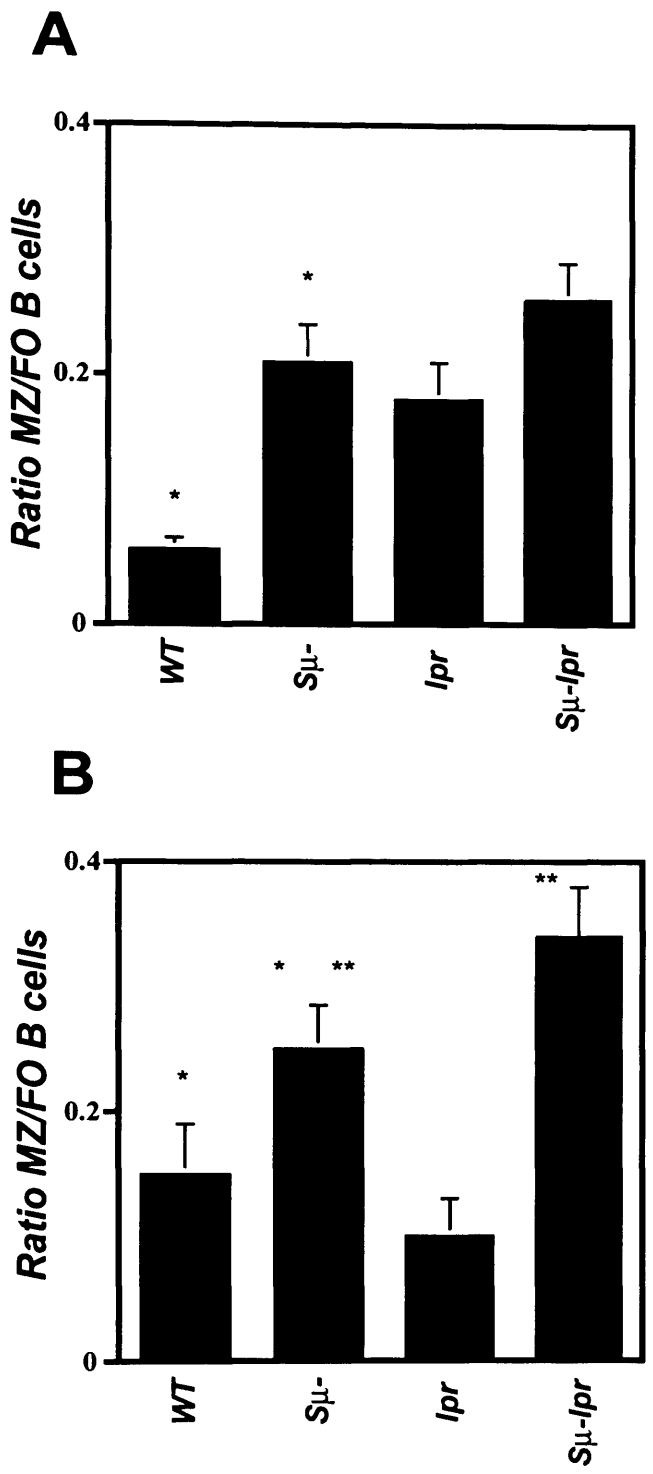

Figure 30. Marginal zone B cells in *Sμ-lpr* and *lpr* mice. Histogram showing MZ:FO B cell ratio derived from FACS analysis. Each bar represents the mean of 6 mice ± SD. (A) Splenic cells from 4 month old mice. *p<0.0001 and **p<0.003 in Student's t-test. (B) Splenic cells from 12 month old mice. *p<0.02 and **p<0.09 in Student's t-test.

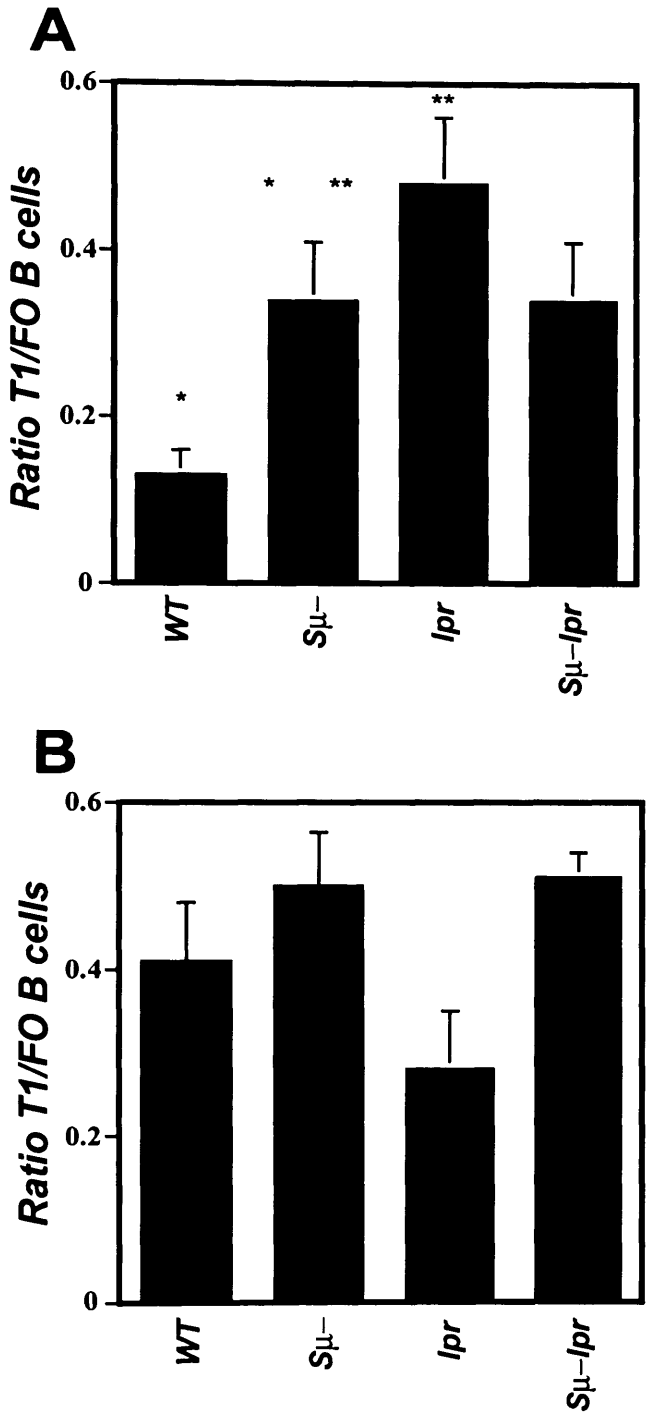

Figure 31. Transitional type 1 B cells in *Sμ-lpr* and *lpr* mice. Histogram showing T1:FO B cell ratio derived from FACS analysis. Each bar represents the mean of 6 mice ± SD. (A) Splenic cells from 4 month old mice. *p<0.0001 and **p<0.04 in Student's t-test. (B) Splenic cells from 12 month old mice.

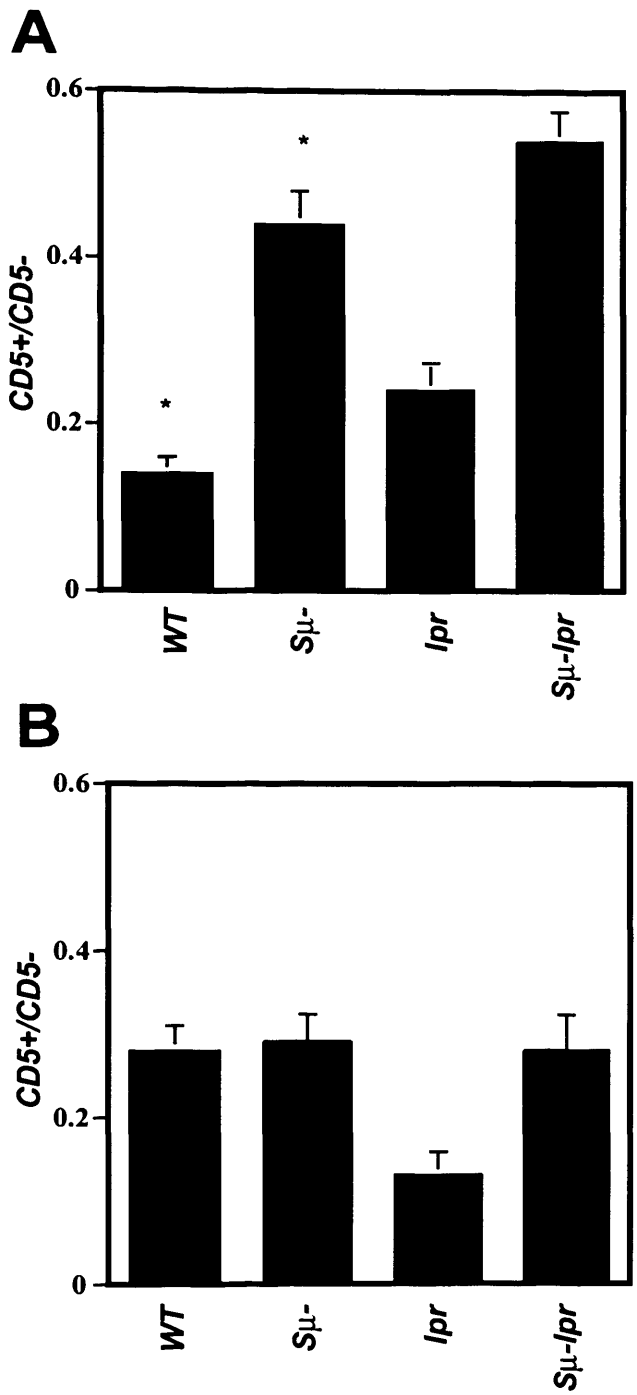

Figure 32. Peritoneal B1 B cells in Sμ-*lpr* and *lpr* mice. Histogram showing CD5+/CD5- B cell ratio derived from FACS analysis. Each bar represents the mean of 6 mice ± SD. *p<0.0007 in Student's t-test. (A) Peritoneal cells from 4 month old mice. (B) Peritoneal cells from 12 month old mice.

DISCUSSION

DISCUSSION

Deficiency of serum IgM leads to an increase in MZ B cells which is reversed by polyclonal, but not monoclonal IgM. I propose that this difference in the effects of these two preparations of IgM is due to the multiple antigen reactivity of polyclonal IgM, acting as an 'adjuvant' to deliver enhanced antigen driven signals to the BCR. Thus, natural IgM acts to regulate B cell selection through, presumably endogenous, antigen mediated BCR signalling.

It has become increasingly clear that antigen interaction is critical for the development of mature B cells arising out of the much larger pool of immature B cells (Allman et al, 1993; Cariappa and Pillai, 2002). It has been suggested that MZ B cells are preferentially formed when BCR signalling is reduced, such as in CD19 deficient mice (Martin and Kearney, 2000). Conversely in the absence of aiolos, which results in enhanced BCR signalling, the formation of follicular B cells is favoured (Cariappa et al, 2001). The pentameric structure of IgM, in contrast to IgG, can potentially yield a high avidity for antigen despite a low antigen specific affinity. Moreover, one molecule of IgM can activate complement whereas two IgG molecules are required (Borsos and Rapp, 1965). Consequently, BCR signalling is reduced and MZ B cells are preferentially formed in the absence of IgM, despite normal levels of serum IgG. A similar process may apply to B1 cells which are also sensitive to antigen drive (Hayakawa et al, 1999),

since polyclonal IgM rather than monoclonal IgM reversed the expansion of this B cell subset.

To test further the effects of serum IgM on B cell selection, a transgenic mouse line that secretes a monoclonal heavy chain (Vμ1) in combination with a variety of light chains was created. In contrast to the injection experiments in Sμ- mice, serum IgM is constitutively secreted in the Vμ1s Sμ- transgenic mice. The effect of the constitutive secretion of Vμ1 on the number of MZ B cells was not different from the Sμ- alone, consistent with the hypothesis that a broad reactivity is needed to influence the mature B cell compartment.

B1 B cell numbers only returned to normal after administration of the polyclonal IgM preparation. B1 B cell numbers were unchanged in Sμ- mice expressing the Vμ1s transgene suggesting that B1 B cells required the full repertoire of polyclonal IgM as well.

The regulation of B1 and MZ B cells is not identical despite the two B cell subsets having some shared functional properties. For example, in the absence of Baff, a member of the TNF superfamily that specifically regulates B lymphocyte proliferation and survival, whereas MZ and follicular B cells are reduced, B1 B cell numbers are unaffected (Schiemann et al, 2001). Similarly, CD22 has opposing effect on B1 and MZ B cells (O'Keefe et al, 1996, Sato et al, 1996a, Samardzic et al, 2002). The response to hapten-Ficoll also divides these two B cell subsets since it is dependent on marginal zone B cells rather than on B1 B cells (Amlot et al, 1985). In Sμ- mice MZ B

cell numbers (but not B1 B cell numbers) further increase with a corresponding reduction in follicular cells in response to PC-Ficoll. When polyclonal IgM is administered during the immune response to PC-Ficoll, the antibody specific titre is diminished. A reduction in MZ B cell numbers towards normal levels when polyclonal IgM is administrated is associated with this lack of responsiveness. Previous data have suggested that serum IgM may inhibit the T-independent immune response (Brodeur and Wortis, 1980). However, it is unclear whether serum IgM is acting through regulating marginal zone B cell numbers or by altering the immunogenicity of the T-independent antigen.

There are also changes in the immature B cell pool in the absence of serum IgM. Previous observations have demonstrated that reduced BCR signaling results in accumulation of immature B cells (Cyster et al, 1996; Turner et al, 1997). Immature transitional type 1 (T1) B cells are the targets of BCR induced negative selection in peripheral B cell development (Su et al, 2002). Serum IgM appears to play a role in this stage of development, since serum IgM deficient mice have increased numbers of T1 B cells, thus negative selection of the T1 compartment may not be sufficient in the absence of serum IgM. Unlike the full reversal of changes in the mature B cell compartment following treatment with polyclonal IgM, the increase in T1 B cells in the spleen is only partially reversed by polyclonal IgM. This may be due to the differential sensitivity of mature and immature B cells to antigen stimulation and that only a fraction of serum IgM is present following

the administration of polyclonal IgM. The relative importance of antigen stimulation/selection as the B cell develops through the various phases of maturation is unclear. Immature B cells are not as dependent on positive selection compared to the mature B cell compartment. Thus in Bruton's tyrosine kinase (Btk) mutant, the principal defect is at the mature B cell stage, immature cells accumulate at the T2 stage with T1 and MZ B cells numbers slightly increased (Loder et al, 1999).

One apparent paradox of these observations is the short time (two weeks) required to reverse the MZ/follicular ratio coupled with the evidence that MZ and FO B cells have a relatively long lifespan (Fulcher and Basten, 1997, Hao and Rajewsky, 2001). This long lifespan however is shortened when immature B cells are differentiating into the mature B cell pool. It is known, and confirmed here, that the MZ/FO ratio does change over a two week period after encountering T-independent antigens and MZ B cells are thought to expand quickly in this scenario (Martin et al, 2001a). Changes in B cell subsets could occur with cell proliferation or apoptosis, or by interchange between the MZ and FO B cell pools. Immature T1 B cell appear to have a lifespan of only a few days. This timeframe probably indicates progression to the mature B cell stage. Higher concentrations of IgM than is obtained after intraperitoneal injections may result in a further reduction in the expanded T1 B cell compartment. Equally, it is possible that the changes in the T1 B cells are in response to the MZ/FO ratio and the

time required for cells to return to normal levels is longer than the two week injection period.

The fact that the administration of monoclonal and polyclonal IgM have different effects, despite achieving the same serum concentration, suggests that the increase in MZ B cells is not due to a feedback mechanism that simply senses the IgM concentration. However, polyclonal IgM may have an advantage even in this situation if a Fcμ receptor binds preferentially to the altered confirmation arising out of IgM complexed with antigen. This process could involve a putative Fcμ receptor, which could act to regulate MZ B cell numbers after binding with serum IgM. Thus, without serum IgM the negative signal from Fcμ would be absent and MZ B cells would expand. This Fcμ receptor would either have to be expressed specifically on B1 and MZ B cells, or that B1 and MZ B cells would be more sensitive to the inhibitory signal delivered by IgM than FO cells. An Fcμ receptor that would specifically down regulate B1 and MZ B cells has not been characterised. However, an Fcμ receptor has been described although this molecule also binds to IgA and is expressed on a comparatively wide range of cells (Sakamoto et al, 2001 and Shibuya et al, 2000). Intriguingly, a recent report has demonstrated that serum IgM is a ligand for CD19 (de Fougerolles et al, 2001), and B1 and MZ B cells are affected by alterations in CD19 expression (Rickert et al, 1995; Sato et al, 1996; Martin and Kearney 2001b).

Cell cycling and apoptosis was studied in Sμ- and control mice. FO B cells from Sμ- mice incorporated more BrdU when mice received continuous treatment of BrdU in the drinking water for 14 days than the control FO B cells, suggesting that FO B cells derived from Sμ- mice are cycling faster. FO B cells also have a shorter lifespan, since they appear to be dying more as assessed by TUNEL. This could explain the reduction in the number of follicular B cells in the Sμ- mice. BrdU labelling has been used extensively to calculate the lifespan of B cells (Rolink et al, 1998) and has shown direct correlation between shortened lifespan and reduced size of the mature B cell population, even when the influx of immature B cells from the bone marrow is normal (Rolink et al, 1999). These results are compatible with the hypotheses that follicular B cells need to receive positive signals through the BCR to maintain homeostasis and that serum IgM contributes to that signal.

There seems to be no intrinsic defects in the B cell population of Sμ- mice, calcium mobilisation, proliferation, and development are normal upon BCR stimulation in vitro. Calcium flux has been implicated in the selection of events that regulate B cell maturation and in antigen receptor triggered proliferation. A number of mutations in signaling molecules have been identified in deficient mice that compromise B cell development and BCR induced calcium release (Healy and Goodnow 1998). Purified immature T2 B cells differentiate into mature FO B cells in vitro in response to BCR crosslinking, but if these cells bear mutations in BCR signalling molecules such as Btk, they fail to differentiate into mature B cells and to induce

calcium flux (Petro et al, 2002 and Genevier et al, 1997). Serum IgM deficient T2 cells differentiate into mature B cells in vitro, demonstrating that immature B cells from serum IgM deficient mice have the ability to differentiate when stimulated.

Marginal zone, B1 and T2 B cells have an activated phenotype indicated by how fast these cells incorporate BrdU over time. MZ and B1 B cells are thought to be self-renewing (Kantor et al, 1993). Experiments where B cell development was blocked by using a inducible RAG-2 transgene, showed that even when there is no influx of B cells from the bone marrow, after 28 weeks MZ and B1 B cells are still found cycling whereas all the other subtypes of B cells have disappeared (Hao and Rajewsky, 2001). MZ and B1 B cells have a more autoreactive repertoire (Oliver et al, 1999 and Hayakawa et al, 1999) and may be constantly activated by self-antigens without the adjuvant effect of serum IgM. In the absence of serum IgM, more self antigens might bind to BCR giving rise to increased numbers of autoreactive MZ and B1 B cells in serum IgM deficient mice.

B1 B cells in the peritoneum behave differently from peritoneal B2 and splenic B1 B cells. They have diminished ability for intracellular calcium mobilisation, aberrant proliferation and increased apoptosis upon BCR crosslinking (Morris et al, 1993). They are also enriched with auto-reactive cells, that may have escaped negative selection in the bone marrow, but once in the periphery these cells could become anergic/hiporesponsive (Chumley et al, 2002 and Dal Porto et al, 2004).

Peritoneal B1 B cells from Sμ- and control mice display a very similar calcium flux response to BCR crosslinking. B1 cells, however from Sμ- mice incorporated less BrdU when mice received continuous treatment of BrdU in the drinking water for 14 days than the control B1 B cells, suggesting that B1 B cells derived from Sμ- mice are cycling slower. B1 B cells also have a longer lifespan, since they appear to be dying less as assessed by TUNEL. This could explain the increase in the number of peritoneal B1 B cells in the Sμ- mice. The mechanisms that govern the survival and homeostasis of MZ and peritoneal B1 B cells are probably different. Natural antibodies (IgM) are produced by B1 B cells in the peritoneal cavity, these antibodies may be very important in controlling the number of B1 B cells down by delivering signals through the BCR, B1 B cells are sensitive to BCR crosslinking and undergo proliferation and apoptosis (Bikah et al, 1996, Morris et al, 1993 and Sen et al, 1999). In the absence of serum IgM B1 B cells proliferate less and live longer, suggesting that these cells are probably more anergic/hyporesponsive than the B1 B cells in the control mice. Serum IgM may be working to "mop up" (auto)antigens and in its absence the (auto)antigens are probably binding directly to the BCR making the B1 B cells more anergic, cycling and dying less.

When serum IgM KO mice were made Fas deficient, by crossing into BL/6 *lpr,* they developed more lymphoproliferation, but no change in autoimmunity occurred. However, IgM deficient MRL*lpr* mice develop a more severe autoimmune phenotype (Boes et al, 2000). Studies in C1q

deficient mice showed that the background is very important in the development of autoimmunity. In BL/6 C1q $^{-/-}$ and BL/6 *lpr* C1q $^{-/-}$ mice there were no evidence of autoimmunity. Mortality was subtlety increased in MRL*lpr* C1q $^{-/-}$ mice, but the degree of autoimmunity was similar to MRL*lpr,* which exhibit a very aggressive early onset of disease (Mitchell et al, 2002).

The more severe lymphoproliferation phenotype in Sμ-*lpr* could be due to the lack serum of IgM combined with the Fas mutation, permitting the survival of autoreactive cells. This would explain my FACS findings of an increase in double negative (DN) T cells and MZ B cells in the spleens of the Sμ-*lpr* mice throughout life compared to *lpr* mice. These populations then decreased with age. The *lpr* mice could compensate for the lack of Fas mediated apoptosis by different mechanisms of cell death, in which IgM is involved. Thus in the absence of circulating IgM, the forming of immune complexes and the clearance of cell debris is thought to be reduced (Kin et al, 2002). IgM plays a very important role in binding to dying cells and is largely responsible and prerequisite for the classical pathway activation mediated by C1q. It was also shown that IgM deficient mice have defective clearance of apoptotic cells by peritoneal macrophages (Quartier et al, 2004).

CONCLUSIONS

CONCLUSIONS

Natural IgM has a wide range of actions in the immune system. The results presented here demonstrate that mice lacking serum IgM have an expansion in marginal zone B cells with a corresponding reduction in follicular B cells. The increase in marginal zone/follicular B cell ratio (and an expansion in peritoneal B1 cells) is fully reversed by administration of polyclonal IgM, but not by a monoclonal IgM preparation. Mice engineered to have a secreted monoclonal IgM repertoire, with an endogenous membrane IgM had a similar marginal zone/follicular ratio and numbers of B1 B cells compared to mice lacking serum IgM. The proposal that natural IgM, by virtue of its polyreactivity, enhances antigen driven signaling through the B cell receptor (BCR) and promotes the formation of B2 and follicular B cells is supported by these results. In particular it is not simply the lack of serum IgM *per se* but the lack of a diverse IgM population.

Splenic follicular B cells have a shortened lifespan whereas peritoneal B1 B cells have a longer lifespan in the absence of serum IgM. However they are fully able to proliferate and to mobilise calcium responses following BCR crosslinking in vitro. These results demonstrate that natural IgM regulates the selection of B lymphocyte subsets in the periphery but may be working in different ways depending on the location/environment.

Splenic B cells may be continuously activated by self-antigens and serum IgM could behave as an adjuvant. Serum IgM complexed with

soluble antigens would be more immunogenic and therefore deliver a stronger signal through the BCR. It is thought that immature B cells require a strong BCR signal to differentiate into follicular B cells, therefore in the absence of serum IgM the signal delivered through the BCR is weak, and MZ B cells are preferentially formed over FO B cells. The reduction in FO B cell numbers in the absence of serum IgM is in accordance with the notion that mature B cells require a certain level of BCR signal strength and that polyclonal IgM is important in increasing antigen immunogenicity. In the peritoneum, natural IgM which is mainly produced by B1 B cells may be working differently than in the spleen. Others have suggested that peritoneal B1 B cells require a strong signal through the BCR. I propose that immunogenic auto-antigens are in greater abundance in the peritoneum compared to the spleen and these Ags are normally "mopped up" by serum IgM reducing their immunogenicity. Thus without serum IgM the increased amounts of peritoneal Ags deliver a stronger signal to B1 B cells resulting in anergy in this B cell compartment.

The observation that only polyclonal IgM (as opposed to monoclonal IgM) is able to downregulate the increased cellular and serological T-independent response seen in serum IgM deficient mice supports the notion that natural IgM exerts its effects through antigen binding. When serum IgM deficient mice were intercrossed with BL/6*lpr* mice no increase in autoimmunity was observed. The lack of acceleration of autoimmunity that would have been predicted by earlier experiments may be due to differences

in the genetic background. The fact that B1 and marginal zone B cells were further expanded in serum IgM BL/6*lpr* mice suggests that the link between these B cell subsets and autoimmunity is not straightforward.

FUTURE WORK

These are the main aims of further work:

• to further investigate the mechanisms that underlie the interplay between natural IgM and B cell subsets, by looking at the immunoglobulin repertoire of immature and mature B cells.

• to study signaling molecules that lead to proliferation (Cyclin D), survival (A1) and apoptosis (caspase 9) in the naïve and activated B cell subtypes.

• to further characterise the two recently described transitional type 2 (T2) subsets, T2 marginal zone precursor (T2-MZP) and T2 follicular precursor (T2-FP).

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APPENDIX

Poster presentations

Natural IgM Regulates B1 and Marginal Zone B Cells-Relationship with Autoimmunity. Baker N and Ehrenstein M. 3rd International Congress on Autoimmunity. Switzerland. Feb 2002

Selection of B Lymphocyte Subsets is Regulated by Natural IgM. Baker N and Ehrenstein M. Keystone Symposia: B Cells and Antibodies: Laboratory to Clinic. USA. Jan 2003

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Selection of B Lymphocyte Subsets is Regulated by Natural IgM. British Society of Immunology. UK. Dec 2002

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